



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US89/01589  <b>(22) International Filing Date:</b> 14 April 1989 (14.04.89)  <b>(30) Priority data:</b> 185,702                              25 April 1988 (25.04.88)              US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US    185,702 (CIP) Filed on                                      25 April 1988 (25.04.88)  <b>(71) Applicant (for all designated States except US):</b> THE RE- GENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive, 22nd Floor, Oakland, CA 94612 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> PARDRIDGE, Willi- am, M. [US/US]; 1180 Tellem Drive, Pacific Palisades, CA 90272 (US). SCHIMMEL, Paul, R. [US/US]; 31 Sol- omon Pierce Road,		Lexington, MA 02173 (US).  <b>(74) Agents:</b> OLDENKAMP, David, J. et al.; Poms, Smith, Lande & Rose, 2121 Avenue of the Stars, Suite 1400, Los Angeles, CA 90067 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (Euro- pean patent), BR, CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), SU, US.  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
<b>(54) Title:</b> CHIMERIC PEPTIDES FOR NEUROPEPTIDE DELIVERY THROUGH THE BLOOD-BRAIN BARRIER  <b>(57) Abstract</b>  <p>Chimeric peptides adapted for delivering neuropharmaceutical agents, such as neuropeptides into the brain by receptor-mediated transcytosis through the blood-brain barrier. The chimeric peptides include a peptide which by itself is capable of crossing the blood-brain barrier by transcytosis at a relatively high rate. The transportable peptide is conjugated to a hydrophilic neuropeptide which by itself is transportable only at a very low rate into the brain across the blood-brain barrier. The resulting chimeric peptide is transported into the brain at a much higher rate than the neuropeptide alone to thereby provide an effective means for introducing hydrophilic neuropeptides into the brain through the blood-brain barrier.</p>		

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CHIMERIC PEPTIDES FOR NEUROPEPTIDE DELIVERY  
THROUGH THE BLOOD-BRAIN BARRIER

BACKGROUND OF THE INVENTION

5       The present invention relates generally to the  
introduction of neuropharmaceutical agents into the  
brain by transcytosis across the blood-brain barrier.  
More particularly, the present invention relates to  
chimeric peptides which are capable of transporting  
10 neuropharmaceutical agents into the brain by receptor-  
mediated transcytosis across the blood-brain barrier.

This invention was made with Government support  
under Grant No. NS-17701 awarded by the National  
Institutes of Health. The Government has certain rights  
15 in this invention. This application is a continuation-  
in-part of copending application Serial No. 06/891,867.

The vertebrate brain has a unique capillary system  
which is unlike that in any other organ in the body.  
The unique capillary system has morphologic characteris-  
20 tics which make up the blood-brain barrier (BBB). The  
blood-brain barrier acts as a systemwide cellular  
membrane which separates the brain interstitial space  
from the blood.

The unique morphologic characteristics of the brain  
25 capillaries which make up the BBB are: (a) epithelial-  
like high resistance tight junctions which literally  
cement all endothelia of brain capillaries together, and  
(b) scanty pinocytosis or transendothelial channels,  
which are abundant in endothelia of peripheral organs.  
30 Due to the unique characteristics of the blood-brain  
barrier, hydrophilic drugs and peptides that readily  
gain access to other tissues in the body are barred from  
entry into the brain or their rates of entry are very  
low.

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Various strategies have been developed for introducing those drugs into the brain which otherwise would not cross the blood-brain barrier. The most widely used strategies involve invasive procedures where the drug is delivered directly into the brain. The most common procedure is the implantation of a catheter into the ventricular system to bypass the blood-brain barrier and deliver the drug directly to the brain. These procedures have been used in the treatment of brain diseases which have a predilection for the meninges, e.g., leukemic involvement of the brain.

Although invasive procedures for the direct delivery of drugs to the brain ventricles have experienced some success, they have not been entirely successful because they only distribute the drug to superficial areas of the brain tissues, and not to the structures deep within the brain. Further, the invasive procedures are potentially harmful to the patient.

Other approaches to circumventing the blood-brain barrier utilize pharmacologic-based procedures involving drug latentiation or the conversion of hydrophilic drugs into lipid-soluble drugs. The majority of the latentiation approaches involve blocking the hydroxyl, carboxyl and primary amine groups on the drug to make it more lipid-soluble and therefore more easily transported across the blood-brain barrier. Although the pharmacologic approaches have been used with some success, they may not be entirely satisfactory for delivery of peptides through the BBB based on the inventor's experience with cyclosporin transport through the BBB. Cyclosporin is a highly latentiated (lipid-soluble) peptide that crosses the BBB relatively slowly.

Another approach to circumventing the blood-brain barrier involves the intra-arterial infusion of hypertonic substances which transiently open the blood-brain barrier to allow passage of hydrophilic drugs. However,

hypertonic substances are potentially toxic and may damage the blood-brain barrier.

There presently is a need to provide improved substances and methods for delivering hydrophilic drugs and peptides across the blood-brain barrier and into the brain. It is desirable that such improved substances and methods provide for uniform introduction of the hydrophilic peptide or drug throughout the brain and present as little risk to the patient as possible.

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#### SUMMARY OF THE INVENTION

In accordance with the present invention, new procedures and substances are disclosed which provide uniform distribution of neuropeptides and other drugs throughout the brain while reducing the problems inherent in prior invasive and pharmacologic drug introduction procedures.

The present invention is based on the surprising discovery that hydrophilic peptides may be physiologically transported across the blood-brain barrier by coupling or conjugating the drug to a transportable peptide which is capable of crossing the blood-brain barrier by receptor-mediated transcytosis. This discovery is particularly surprising in view of the traditional notion that the blood-brain barrier is a passive barrier which is impenetrable by hydrophilic drugs or peptides.

The invention involves novel chimeric peptides which are adapted to deliver a neuropharmaceutical agent into the brain by transcytosis across the blood-brain barrier. The chimeric peptides include a transportable peptide that is capable of crossing the blood-brain barrier at relatively high rate by receptor-mediated transcytosis. The transportable peptide is conjugated with a neuropharmaceutical agent to form the chimeric peptide. The neuropharmaceutical agent is generally a

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hydrophilic peptide that does not by itself significantly cross the BBB. The conjugation of transportable peptides with neuropharmaceutical agents was surprisingly found to produce chimeric peptides which were capable  
5 of being transported across the blood-brain barrier.

Histones are a group of naturally occurring proteins which have been found to be well suited for use as a transportable peptide in accordance with the present invention. Since histones are naturally  
10 occurring substances, they do not require organic synthesis and the possibility of an immune response associated with synthetically derived materials is greatly reduced.

The chimeric peptides are believed to be transported across the blood-brain barrier by the physiologic process of transcytosis via receptors in the blood-brain barrier. This insures that the chimeric peptide is distributed uniformly to all parts of the brain. In addition, the introduction of the chimeric peptide into  
15 the brain by a physiologic pathway reduces the harmful side effects and risks inherent in the traditional invasive and pharmacological approaches.

The present invention also includes methods for administering the chimeric peptides subcutaneously or  
25 intranasally and the chimeric peptide containing compositions utilized in such methods of treatment.

The above-discussed and many other features and attendant advantages of the present invention will become apparent as the invention becomes better understood by reference to the following detailed description when considered in conjunction with the accompanying  
30 drawing.

#### BRIEF DESCRIPTION OF THE DRAWING

35 Fig. 1 is a chart showing the results of the tests described in Example No. 2.

Fig. 2 is a chart depicting the results of tests in accordance with example 9 showing the uptake of histone by brain capillaries.

Fig. 3 is a chart depicting the results of histone uptake tests in accordance with example 9 which show the temperature and time dependence of the transport mechanism.

Fig. 4 is a chart showing the linearity of histone with respect to the amount of capillary protein.

Fig. 5 is a chart showing the total and acid resistant binding of [ $^{125}\text{I}$ ]-histone plotted versus time at 37°C and 4°C incubations.

Fig. 6 depicts charts wherein the binding (% bound/mg<sub>p</sub>) of [ $^{125}\text{I}$ ]-histone is plotted versus the concentration of unlabeled histone in the incubation vessel and wherein bound (B)/free (F) is plotted versus the [ $^{125}\text{I}$ ]-histone bound to the bovine brain capillaries (nmol/mg<sub>p</sub>).

Fig. 7 is a chart showing the serum [ $^3\text{H}$ ]-albumin and [ $^{125}\text{I}$ ]-histone radioactivity, A(t), (DPM/ml/percent injected) plotted versus time after a single intravenous injection of both isotopes.

#### DETAILED DESCRIPTION OF THE INVENTION

The chimeric peptides in accordance with the present invention are useful in delivering a wide variety of neuropharmaceutical agents to the brain. The invention is particularly well suited for delivering neuropharmaceutical agents which are hydrophilic peptides. These hydrophilic peptides are generally not transported across the blood-brain barrier to any significant degree.

Exemplary hydrophilic peptide neuropharmaceutical agents are: thyrotropin releasing hormone (TRH) - used to treat spinal cord injury and Lou Gehrig's disease; vasopressin - used to treat amnesia; alpha interferon-

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used to treat multiple sclerosis; somatostatin - used to treat Alzheimer's disease; endorphin - used to treat pain; L-methionyl (sulfone)-L-glutamyl-L-histidyl-L-phenylalanyl-D-lysyl-L-phenylalanine (an analogue of adrenocorticotrophic hormone (ACTH)-4-9) - used to treat epilepsy; and muramyl dipeptide - used to treat insomnia. All of these neuropharmaceutical peptides are available commercially or they may be isolated from natural sources by well-known techniques.

The following description will be limited to chimeric peptides in which the neuropharmaceutical agents are hydrophilic peptides (neuropeptides) with it being understood that the invention has application to any neuropharmaceutical agent which by itself is transported at a low or non-existent rate across the blood-brain barrier. The invention also has application where it is desired to increase the rate at which the neuropharmaceutical agent is transported across the blood-brain barrier.

The chimeric peptide includes the hydrophilic peptide drug conjugated to a transportable peptide which is capable of crossing the blood-brain barrier by transcytosis at a much higher rate than the hydrophilic neuropeptides. Suitable transportable peptides include: histone, insulin, transferrin, insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), basic albumin and prolactin.

Transferrin is an 80K glycoprotein that is the principal iron transport protein in the circulation. Transferrin is also a protein that is enriched in the cerebrospinal fluid (CSF). Transferrin is widely available and may be purchased or isolated from blood or CSF by well-known procedures.

Insulin, IGF-I and IGF-II are also commonly available. Insulin is available on a wide scale commercially and may also be recovered from natural



sources by well-known techniques. IGF-I and IGF-II are available from commercial outlets such as Amgen or Peninsula Labs or they may be isolated from natural sources according to the procedure of Rosenfeld et al. (J. Clin Endocrinol. Metab. 55, 434, 1982).

Basic albumin or cationized albumin has a isoelectric point (pI) of 8.5 as compared to a pI of 3.9 for natural albumin. Cationized albumin, unlike natural albumin, enters the brain rapidly across the blood-brain barrier. Cationized albumin (pI = 8.5) is prepared preferably by covalent coupling of hexamethylene-diamine (HMD) to bovine serum albumin (pI = 3.5) according to Bergmann, et al., "Cationized Serum Albumin Enhances Response of Cultured Fetal Rat Long Bones To Parathyroid Hormone", Endocrinology, 116:1729-1733 (1985). An exemplary synthesis is as follows: 10 ml of a 10% solution of albumin in water is slowly added to 60 ml of 2.0 M HMD and the pH of the solution is adjusted to 6-7 with 1N HCl. After 30 minutes, 1 g of N-ethyl-N'-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDAC) is added to activate the carboxyl groups of the albumin, followed by the addition of another 1 g EDAC 1 hour later. The pH is constantly adjusted to 6-7 with 0.2N HCl. The solution is allowed to stand overnight with constant stirring. The next day the solution is dialyzed extensively against distilled water. This solution is then purified by chromatofocusing using the Pharmacia polybuffer exchanger 94 resin and the polybuffer 96 elution buffer.

Histone is especially well suited for use as a transportable peptide because it is a naturally occurring protein that does not require organic synthesis such as the above procedure for preparing basic albumin. Further, the absence of antibody response to the naturally occurring histone makes it suitable in many situations where immune responses to synthesized

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materials, such as cationized albumin, would potentially limit its utility. Histones are a group of lysine-rich, highly cationic proteins that are subdivided into five classes (H1, H2, H3, H4, and H5). There are multiple subtypes in each of the five classes. These proteins are found in the nucleus of all cells and are tightly bound to the phosphate groups of chromatin. The histone molecules play a vital role in chromatin organization. The histones are routinely isolated from the acid-soluble fraction of nuclei isolated from calf thymus, chicken erythrocytes, or other starting materials. The different subtypes are separated by a number of well known techniques, such as isoelectric focusing or ion-exchange chromatography.

One characteristic of the histone molecules which is important for linkage chemistry is that the histones, with the exception of H3, lack a cysteine sulfhydryl group. Histones are known to undergo an extensive number of chemical modifications within the normal cell that include N-methylation, O-methylation, acetylation, phosphorylation, adenosine diphosphate (ADP) ribosylation, ubiquitination, and enzymatic hydrolysis of specific peptide bonds.

Histones are available from a wide variety of commercial sources or they may be isolated according to known procedures set forth in the following references:

Wu, R.S., Panusz, H.T., Hatch, C.L., and Bonner, W.M. (1986): Histones and their modifications. CRC Crit. Rev. Biochem. 20: 201-263; and

Coles, L.S., Robins, A.J., Madley, L.K., and Wells, J.R.E. (1987): Characterization of the chicken histone H1 gene complement. J. Biol. Chem. 262: 9656-9663.

Histones isolated from any of the conventional sources may be used and the particular class or subtype

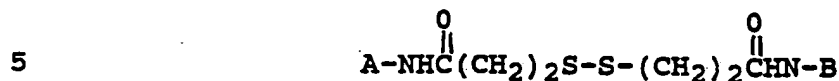
is also not particularly critical. It is preferred that histones isolated from human sources be used for preparing chimeric peptides for use in treating humans.

Another suitable transportable peptide is prolactin. Prolactin is a hormone which is secreted by the anterior pituitary. Prolactin is widely available commercially or it can be isolated from pituitary glands by well-known procedures.

The chimeric peptides are made by conjugating a transportable peptide with the neuropharmaceutical peptide. The conjugation may be carried out using bifunctional reagents which are capable of reacting with each of the peptides and forming a bridge between the two. The preferred method of conjugation involves peptide thiolation wherein the two peptides are treated with a reagent such as N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) to form a disulfide bridge between the two peptides to form the chimeric peptide. Other known conjugation agents may be used, so long as they provide linkage of the two peptides (i.e. the hydrophilic peptide drug and the transportable peptide) together without denaturing them. Preferably, the linkage can be easily broken once the chimeric peptide has entered the brain. Suitable examples of conjugation reagents include: glutaraldehyde and cystamine and EDAC. Conjugation of peptides using glutaraldehyde is described in Poznansky et al., Insulin: Carrier potential for enzyme and drug therapy. Science 223:1304-1306, 1984. Conjugation of peptides using cystamine and EDAC is described in Ito et al., Transmembrane delivery of polypeptide hormones bypassing the intrinsic cell surface receptors: a conjugate of insulin with  $\alpha$ 2-macroglobulin ( $\alpha$ 2M) recognizing both insulin and  $\alpha$ 2M receptors and its biological activity in relation to endocytic pathways. Mol Cell Endocrinol 36:165, 1984.

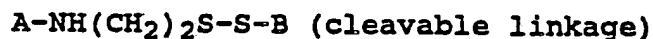
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Examples of preferred chimeric peptides include those having the general structure

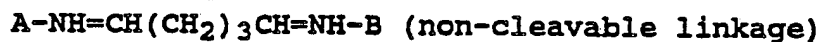


where A is somatostatin, thyrotropin releasing hormone (TRH), vasopressin, alpha interferon, endorphin, muramyl dipeptide or ACTH 4-9 analogue; and B is histone, insulin, IGF-I, IGF-II, transferrin, cationized (basic) albumin or prolactin.

Other examples of preferred chimeric peptides include those listed above wherein the disulfide conjugating bridge between A and B is replaced with bridges having the following structures:



which are formed when cystamine and EDAC are employed as the bridge reagents;



which are formed when glutaraldehyde is employed as bridge reagent.

The chimeric peptides can be introduced into the body by any conventional procedure including parenteral injection or intranasal inhalation. Preferably, the chimeric peptides are combined with a compatible pharmaceutical carrier and injected parenterally or if desired combined with a suitable carrier and administered intranasally in accordance with the well-known conventional procedures used for intranasal administration of insulin. Suitable carrier solutions include those commonly used in injectable or nasal-inhaled hormone preparations such as sterile saline at a pH of

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around 5 which includes common bacteriostatic agents. The concentration of a chimeric peptide in the carrier will vary depending upon the specific transportable peptide and the specific neuropharmaceutical peptide.

5 Preferably, levels of the chimeric peptide in the carrier should be between about 0.001 weight percent to 0.01 weight percent. As a general rule, the dosage levels and percent of chimeric peptides present in the injection or intranasal solution should correspond to

10 the accepted and established dosages for the particular neuropharmaceutical peptide as well as the transportable peptide.

Examples of practice are as follows:

15 Example 1 - Synthesis of Somatostatin-Insulin Chimera

Somatostatin, a peptide deficient in the brain of Alzheimer's disease, is a peptide which is not transported through the blood-brain barrier. Conversely, insulin is a peptide that is transported through the

20 blood-brain barrier. The transportability of insulin through the blood-brain barrier is set forth in my article entitled "Receptor-Mediated Peptide Transport Through The Blood-Brain Barrier" (Endocrine Reviews, Vol. 7, No. 3, August 1986), the contents of which is

25 hereby incorporated by reference.

Somatostatin and insulin were conjugated by peptide thiolation using a reversible peptide-peptide conjugation method as described by Carlsson, et al. in "Protein Thiolation and Reversible Protein-Protein Conjugation"

30 (Biochem. J. (1978) 173, 723-737). A heterobifunctional reagent, N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), was used to couple a lysine or free N-terminus on insulin to a free lysine or amino terminus on somatostatin. Approximately 0.3 mg of insulin and 26 uCi of

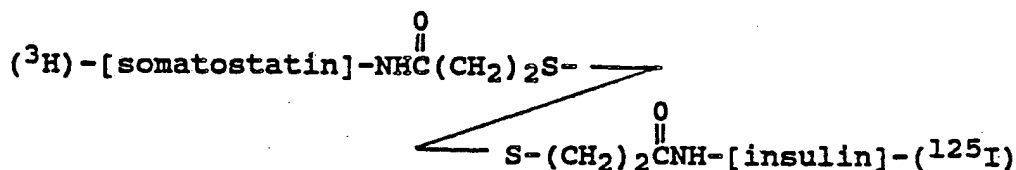
35  $^{125}\text{I}$ -insulin in 2 ml of phosphate buffered saline was prepared. To half of this was added 4 lambdas of 20 mM

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fresh SPDP and this was incubated at room temperature for 45 minutes.

Separately, 180 uCi of tritiated somatostatin in 180 uL of 0.01 N HCl was solubilized and added to 180 uL of 0.2 M phosphate buffered saline. To half of this, 4 uL of 20 mM SPDP was added and this was incubated for 45 minutes, followed by acidification with 20 uL of 0.75 M sodium acetate (pH = 4.5) followed by reduction with 20 uL of 0.25 M dithiothreitol. This was incubated at room temperature for 30 minutes followed by brief dialysis to remove unreacted small molecules. The conjugated insulin and conjugated somatostatin were then incubated overnight at room temperature followed by dialysis and counting for tritium and  $^{125}\text{I}$  radioactivity. This resulted in a total of 53 uCi of  $^3\text{H}$ -somatostatin coupled to 5.3 uCi of  $^{125}\text{I}$ -insulin in 2 ml of phosphate buffered saline.

The structure of the somatostatin-insulin chimera is shown below.



Somatostatin has the following amino acid sequence: Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys. Insulin is a double chain protein hormone whose structure is well known.

Example 2 - Radioreceptor Assay Using Isolated Bovine Brain Microvessels and  $^3\text{H}$ -Somatostatin- $^{125}\text{I}$ -Insulin Chimera

Somatostatin was obtained from Peninsula Laboratories and tritiated by reductive methylation using  $^3\text{H}$ -sodium borohydride. Insulin was obtained from Sigma

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Chemical Company and was iodinated by oxidative iodination using chloramine T and  $^{125}\text{I}$ -iodine. The two compounds were coupled together using SPDP as described in Example 1. Bovine brain microvessels were isolated  
5 as described in Pardridge, et al., "Rapid Sequestration And Degradation Of Somatostatin Analogues By Isolated Brain Microvessels", (Journal of Neurochemistry, Vol. 44, No. 4, 1985, pp. 1178-1184).

$^3\text{H}$ -somatostatin was added to one set of micro-  
10 vessels for up to 60 minutes incubation at room temperature. In another set of incubations, the  $^3\text{H}$ -somatostatin- $^{125}\text{I}$ -insulin chimera was also added. As shown in Fig. 1, the uptake of the chimera was more than double that of the free somatostatin. Moreover, the uptake of  
15 the chimera increased with time, whereas there was no increase in time with the free somatostatin. The uptake of the free somatostatin likely represents nonspecific binding as described in the article mentioned above (Journal of Neurochemistry, Vol. 44, No. 4, 1985).

20 This example demonstrates the receptor-mediated transcytosis or endocytosis of somatostatin-insulin chimera via the insulin receptor. Previous studies have shown that the receptor-mediated endocytosis of peptides in the isolated brain microvessels is a reliable index  
25 of the in vivo blood-brain barrier receptor transport activity of peptides in vivo (see my previously-mentioned article in Endocrine Reviews, Vol. 7, No. 3, August 1986).

30 Example 3:

A chimeric peptide is prepared according to the same procedure as in Example 1 except that transferrin is substituted for insulin. The resulting chimeric peptide is combined with sterile saline to provide a  
35 solution containing 0.01 weight percent chimeric peptide which is administered to the patient parenterally or

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intranasally.

Example 4:

5 A chimeric peptide is prepared according to the same procedure as in Example 1 except that vasopressin is substituted for somatostatin. The resulting chimeric peptide is combined with sterile saline to provide a solution containing 0.01 weight percent chimeric peptide which is administered to the patient parenterally.

10

Example 5:

15 A chimeric peptide is prepared according to the same procedure as in Example 1 except that transferrin is coupled to alpha-interferon. The resulting chimeric peptide is combined with sterile saline to provide a solution containing 0.01 weight percent chimeric peptide which is administered to the patient or subject parenterally or intranasally.

20 Example 6:

A chimeric peptide is prepared according to the same procedure as in Example 1 except that IGF-II is coupled to beta-endorphin. The resulting chimeric peptide is combined with sterile saline to provide a solution containing 0.01 weight percent chimeric peptide which is administered to the patient or subject parenterally or intranasally.

25

Example 7:

30 A chimeric peptide is prepared according to the same procedure as in Example 1 except that insulin is coupled to ACTH 4-9 analogue. The resulting chimeric peptide is combined with sterile saline to provide a solution containing 0.01 weight percent chimeric peptide which is administered to the patient or subject parenterally or intranasally.

35



Example 8:

A chimeric peptide is prepared according to the same procedure as in Example 1 except that cationized albumin is coupled to hexosaminidase A. The resulting  
5 chimeric peptide is combined with sterile saline to provide a solution containing 0.01 weight percent chimeric peptide which is administered to the patient or subject parenterally or intranasally.

10 Example 9:

A chimeric peptide is prepared according to the same procedure as in Example 1 except that commercially available bovine histone type V is substituted for insulin. The resulting chimeric peptide is combined  
15 with sterile saline to provide a solution containing 0.01 weight percent chimeric peptide which is administered to the patient parenterally or intranasally.

Example 10:

20 To demonstrate the usefulness of histone as a polycationic transportable peptide, the following uptake tests were conducted.

A radio receptor assay with bovine brain capillaries was conducted according to the procedure set forth in Example 2, except that commercially supplied  
25 bovine histone type V, instead of the somatostatin-insulin chimera, was radiolabeled with  $^{125}\text{I}$ -iodine and chloramine-T. The histone was incubated with the brain capillaries for 60 minutes and the percent uptake per mg  
30 brain capillaries was determined. The results of these tests are depicted in Fig. 2 and they show that the uptake of the histone was high, approximating 110% bound per mg protein (background binding is 3-5% per mg protein). Also, it was found and Fig. 2 shows that the  
35 uptake of the histone is readily saturable by increasing concentrations of unlabeled histone and that the 50%

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inhibition point is reached at a concentration of 300 ug/ml or approximately 14 uM. This saturation response is typical of a receptor-mediated or absorptive-mediated endocytosis mechanism.

5       The amount of histone V uptake was measured at 2, 10, 30 and 60 minute intervals. It was found that the uptake of the histone increases with time as shown in Fig. 3. Incubations of histone V were conducted at 37°C. and 4°C. Histone uptake was inhibited at 4°C. as  
10 also shown in Fig. 3. Further, histone uptake resistance to acid wash was measured by treating the bovine brain capillaries with a mild acid wash after histone uptake was completed. The acid wash was accomplished by rinsing the brain capillaries in a solution of cold  
15 0.028 molar sodium acetate, 0.02 molar sodium barbital and 0.12 molar sodium chloride having a pH of about 3.0. The results of these tests showed that the uptake of histone by the isolated brain capillaries is partially resistant to the mild acid wash. See the graphic  
20 results in Fig. 3 showing acid resistant histone uptake. The results indicate that about one-third of the histone taken up is actually endocytosed into the brain capillaries. The above results showing that both binding and endocytosis are slowed by incubation at 4°C. is typical  
25 of a receptor mediated-mediated or adsorptive-mediated uptake mechanism for transport across the blood-brain barrier.

A one nanomolar concentration of the  $^{125}\text{I}$ -histone V was incubated at room temperature for 10 minutes in the  
30 presence of brain capillaries. The amount of labeled histone taken up by the brain capillaries was then determined. The results of the tests showed that the uptake of the  $^{125}\text{I}$ -histone V is linear with respect to the amount of capillary protein in the incubation flask.  
35 The results of these tests are shown graphically in Fig. 4.

Example 11 -

This example demonstrates the preparation of a histone/horseradish peroxidase conjugate which was tested and shown to be sequestered by brain microvas-  
5 culature. The histone/horseradish peroxidase conjugate was prepared as follows.

Sulfhydryl groups were introduced onto histone by a modification of the procedures described by Jue et al. (1978) Biochemistry 17:5399-5405. Sixteen milligrams of  
10 histone (Sigma type II-AS) was dissolved in 3.0 ml of 50 mM triethanolamine buffer, pH 8.25, in a sealed reaction vessel. The protein solution was exhaustively degassed and purged with nitrogen. To this was added 0.074 ml of a 0.5 M solution of 2-iminothiolane (Pierce) via  
15 syringe. The mixture was allowed to react for 45 minutes at 25°C. In order to reduce all sulfhydryls, the product was then incubated with 0.5 ml of a 0.1 M dithiothreitol (Calbiochem) for 30 min. at 37°C with mild shaking (75 rpm). The modified protein was  
20 purified from the reaction mixture by chromatography over Sephadex G-25 (1.5 x 30 cm) using degassed, nitrogen purged triethanolamine buffer. Protein elution was monitored by UV absorbance at 280 nm [5-5'Dithio-  
25 Biophys. 82:70-77]. Typically, 1.1 moles of sulfhydryl-/mole of protein were introduced using these conditions.

Horseradish peroxidase (HRP) was derivatized with maleimide groups using N-γ-maleimidobutyryloxysuccinimide (GMBS) by a method described by Hashida et al.  
30 (1984) J. Appl. Biochem. 6:56-63. To 20 mg of peroxidase (0.5 mmoles, Boeringer Mannheim EIA grade) was added 1.5 ml of 0.1 M sodium phosphate buffer, pH 7.0. Sixteen mg (57 mmoles) GMBS was dissolved in 0.2 ml of DMF and added to the peroxidase solution. The mixture  
35 was shaken for 30 minutes at 30°C. Derivatized HRP was isolated by chromatography of the reaction mixture over

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Sephadex G-25 (1.5 x 30 cm) using 0.1 M phosphate, pH 6 as an eluant. HRP elution was monitored by its absorbance at 403 nm.

5 The pH of the thiolated histone solution was adjusted to 6 with 1 N HCl. This was immediately added to the maleimide derivatized HRP. The solution was exhaustively degassed, nitrogen purged and septum sealed. The components were allowed to react for 48 hours at 25°C. Separation of the conjugate from  
10 unreacted proteins was achieved on a Sephadex G 100 SF column (2.5 x 54 cm) using 0.1 M phosphate, pH 6 as an eluant. Fractions isolated from the column were checked for absorbance at 230 nm and 403 nm. The first absorbing eluant was pooled, dialyzed against water at 4°C  
15 overnight and lyophilized. Following reconstitution in 2 mL of PBS, 80% (16 mg) of the peroxidase was isolated with the conjugate peak. SDS-page analysis on the conjugate revealed several bands of MW 54 kD and above and could not detect any free HRP or histone. Further-  
20 more, total protein, as determined by the method of Lowry, indicated 1/3 of the mass could be accounted for by HRP suggesting at 6/1 histone/HRP conjugation ratio. The conjugate was adjusted to 1 mg/ml (total protein by Lowry method) and stored at 4°C prior to in vivo  
25 analysis.

The histone/HRP conjugate was tested as follows:

BALB/C mice (6 weeks, female) were injected intravenously with 0.2 ml of histone/HRP conjugate in a  
30 phosphate buffered saline (PBS). Animals were killed after 15 minutes with a lethal injection of chlorohydrate, perfused with 5.0 ml of PBS containing 4% paraformaldehyde, and then the brain was removed and placed into fixative for an additional 15 minutes.  
35 Frozen sections (30 microns thick) were prepared and floated in PBS for at least 20 minutes. The sections

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were removed from PBS and placed in incubation solution [20 mM sodium acetate, pH 3.3, 2.5% ethanol, 4mM sodium nitroprusside, 250 mM 3,3',5,5'- tetramethyl benzidine (TMB)] for 20 minutes. The reaction was initiated by the addition of 1 ml of 0.3% H<sub>2</sub>O<sub>2</sub> to each 100 ml of incubation solution. The reaction was allowed to continue for 10 minutes at room temperature and then the sections were transferred to 20 mM sodium acetate pH 3.3. The sections were washed six times for five minutes each. Tissue sections were mounted onto gelatin coated slides and allowed to dry for seven hours at room temperature. The slides were heated at 60°C for 1 hour, and stained for 30 seconds with 0.5% toluidine blue pH 4.5. The slides were dehydrated in a graded alcohol series, washed with xylene, and mounted with Permount.

The capillaries of the brain were visualized by the presence of the TMB reaction product associated with the luminal surface of the capillary. The product was observed in animals injected with the histone/HRP conjugate, but was not observed in animals injected with either HRP alone or in PBS injected animals. Both white and gray matter were labeled with TMB. There was no overt appearance of specific localization to only highly vascularized areas of the brain.

25

#### Example 12 -

This example sets forth experiments which demonstrate that histone is capable of penetrating the blood-brain barrier (BBB) in vivo. Calfthymus histone was iodinated with [<sup>125</sup>I]-iodine and was found to be rapidly taken up by isolated bovine brain capillaries used as an in vitro model system of the BBB via a time- and temperature-dependent mechanism. The binding was saturable and a Scatchard plot of the binding data was linear, yielding a K<sub>D</sub> = 15.2 ± 2.8 μM and a maximal binding (B<sub>max</sub>) = 7.7 ± 1.0 nmol/mg protein (Fig. 5).

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Other polycations such as protamine or polylysine markedly inhibited uptake of [ $^{125}\text{I}$ ]-histone, but cationized albumin demonstrated minimal inhibition and cationized immunoglobulin caused no inhibition of bovine brain capillary uptake of [ $^{125}\text{I}$ ]-histone. The in vivo brain volume of distribution of [ $^{125}\text{I}$ ]-histone reached  $159 \pm 70 \mu\text{L/g}$  by ten minutes of carotid arterial perfusion as compared to the 10 minute volume of distribution for [ $^3\text{H}$ ]-albumin,  $17 \pm 7 \mu\text{L/g}$ . Most of this uptake represented sequestration by the vasculature, but approximately 8% of the total histone taken up by brain was found to be transported unmetabolized (based on trichloroacetic acid (TCA) precipitability) into brain interstitium.

The experiments were conducted as follows:

#### METHODS

##### Materials

[ $^{125}\text{I}$ ]-iodine was obtained from DuPont-New England Nuclear Corporation (Boston, MA). [ $^3\text{H}$ ]- $\text{NaBH}_4$  was purchased from Amersham Corporation (Chicago, IL). Bovine albumin (Pentex fraction V) was obtained from Miles Laboratories (Elkhart, IN). Male, Sprague-Dawley rats (200-300 g) were obtained from Bantin and Kingman (Fremont, CA). Calf thymus histone VS (lysine-rich) and all other reagents were obtained from Sigma Chemical Company (St. Louis, MO).

##### Histone Iodination

Histone was iodinated to a specific activity of 10-20  $\mu\text{Ci}/\mu\text{g}$  using [ $^{125}\text{I}$ ]-iodine and chloramine T. Fifty  $\mu\text{g}$  of histone (2.3 nmol) were reacted with 2.5 mCi of [ $^{125}\text{I}$ ]-iodine (1.2 nmol) and 2.1 nmol chloramine T followed by a 60 second incubation at room temperature. The mixture was acidified with 0.01 N HCl and applied to an 0.7 x 28 cm column of Sephadex G-25 (medium) and 0.01 N HCl. The iodinated histone eluted in the void volume and was 98% precipitable with trichloroacetic acid

(TCA). The [ $^{125}\text{I}$ ]-histone was stored at 4°C in 0.01 N HCl, but was subject to relatively rapid de-iodination over the course of a week. Therefore, the in vivo carotid artery perfusion experiments were performed with  
5 24 hours of iodination, and the isolated capillary experiments were performed with 3 to 4 days of iodination.

#### Tritiation of Albumin

Bovine albumin (Pentex fraction V) was tritiated to  
10 a specific activity of 0.4  $\mu\text{Ci}/\mu\text{g}$  with [ $^3\text{H}$ ]-NaBH<sub>4</sub> as described previously (Pardridge et al., 1985a). The TCA precipitability of this preparation was >99%.

#### Brain Microvessel Experiments

Bovine brain microvessels were isolated with a  
15 mechanical homogenization technique, as described previously (Pardridge et al.: Rapid sequestration and degradation of somatostatin analogues by isolated brain microvessels. J. Neurochem. 44: 1178-1184, 1985) from fresh bovine cortex obtained from a local slaughter-  
20 house. The final microvessel pellet was cryopreserved in 0.28 M sucrose, 0.02 M Tris (pH 7.4) and 2 mM dithiothreitol in liquid nitrogen (-70°C). On the day of the experiment, the microvessels were thawed, centrifuged, and resuspended in Ringer-HEPES buffer  
25 (RHB) (10 mM HEPES, pH 7.4, 141 mM NaCl, 4 mM KCl, 2.8 mM CaCl<sub>2</sub> and 0.1 gm/dl bovine serum albumin). Uptake of [ $^{125}\text{I}$ ]-histone by bovine brain microvessels was performed as described previously (Pardridge et al.: Cationization of immunoglobulin G (IgG) as a new  
30 strategy for enhanced IgG delivery through the blood-brain barrier. Clin. Res. 37: 140A). Briefly, approximately 100  $\mu\text{g}$  of capillary protein was incubated with 0.2  $\mu\text{Ci}/\text{ml}$  of [ $^{125}\text{I}$ ]-histone in a final volume of 0.45 ml RHB at 37°C or 4°C for time periods ranging from 5  
35 seconds to 60 minutes. Competitive binding studies were performed by adding various concentrations of either

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unlabeled histone, cationized immunoglobulin, native or cationized bovine serum albumin, protamine, or polylysine (59,000 molecular weight). At the end of the incubation period, the mixture was centrifuged at 10,000 g for 45 seconds and the capillary pellet was solubilized in 0.5 ml of 1 N NaOH, followed by [ $^{125}$ I] counting and protein determination by the method of Lowry et al. (Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 262-275, 1951).

Internalization of the labeled histone by the isolated bovine brain capillaries was assessed by a mild acid wash assay as described previously (Pardridge et al., Rapid sequestration and degradation of somatostatin analogues by isolated brain microvessels. J. Neurochem. 44: 1178-1184, 1985). The acid wash solution consisted of 0.12 M NaCl, 0.02 M sodium acetate (pH = 3) and 0.028 M of sodium barbital.

#### [ $^{125}$ I]-Histone Transport Through the BBB In Vivo

Quantitation of in vivo transport of [ $^{125}$ I]-histone through the BBB in vivo was determined with an internal carotid artery perfusion technique coupled with a capillary depletion method. Rats were anesthetized with ketamine/xylazine (ketamine, 200 mg/kg, i.p./xylazine, 2 mg/kg, i.p.) and, following exposure of the right common carotid artery, the occipital, superior thyroid, and pterygopalatine arteries were closed by electrocoagulation. The right external carotid artery was catheterized with a polyethylene catheter (PE-10). The common carotid artery was tied just before the perfusion was started and was kept closed. The perfusion consisted of Krebs-Henseleit buffer, pH 7.4 (118 mM NaCl, 4.7 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 25 mM  $\text{NaHCO}_3$ , 10 mM D-glucose and 3 gm/dl bovine serum albumin), containing 2.5  $\mu\text{Ci/ml}$  of [ $^{125}$ I]-histone and 25  $\mu\text{Ci/ml}$  of [ $^3\text{H}$ ]-albumin. The perfusate was maintained at 37°C and was continuously oxygenated during the perfusion, which



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was carried out at a 1-1.2 ml/min flow rate (Harvard peristaltic pump Model 1210) for 1 to 10 minutes. For perfusion times longer than 2.5 minutes, the rat blood volume was maintained constant by withdrawing blood from  
5 the femoral artery (through a PE-50 catheter filled with heparin) at the same flow rate (Harvard syringe pump Model 940). Following the perfusion, the animals were decapitated and the ipsilateral brain hemisphere was removed. The choroid plexus was discarded, and the  
10 brain was weighed and then homogenized in 3.5 ml of physiologic buffer, pH = 7.4 (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM D-glucose). Four ml of 26% dextran solution (79,000 molecular weight) was added to a final dextran concentration of 13%, and the material was re-homogenized  
15 (3 strokes). All of the homogenization procedures were performed at 4°C.

After removing an aliquot of the homogenate for radioisotope counting, the remainder was centrifuged at  
20 5,400 g for 15 minutes at 4°C in a swinging bucket rotor (Beckman JA-7.5 rotor, Beckman J2-21 centrifuge). The supernatant and pellet were carefully separated. Microscopic examination of the pellet showed that it consisted of brain vasculature, red cells, and brain  
25 nuclei, whereas the supernatant was essentially devoid of vasculature. Aliquots of the supernatant fraction and perfusate (to which was added an amount of dextran similar to that present in the supernatant) were taken for 25% TCA precipitability. Homogenate, pellet,  
30 supernatant, and perfusate samples were solubilized in 2 ml Soluene-350 (Packard Instrument Co., Downers Grove, IL) and prepared for [<sup>125</sup>I], [<sup>3</sup>H] double isotope liquid scintillation spectrometry as described previously (Pardridge, Carrier-mediated transport of thyroid  
35 hormones through the blood-brain barrier. Primary role of albumin-bound hormone. Endocrinology 105: 605-612,

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1979.)

Volumes of distribution for both the [ $^{125}\text{I}$ ]-and the [ $^3\text{H}$ ]-labeled proteins were calculated for the homogenate, pellet, and the postvascular supernatant:

5

$$V_D = \frac{\text{DPM-f/g tissue}}{\text{DPM-ml perfusate}}$$

10 where DPM-f = the DPM in the respective fraction (i.e., homogenate, pellet, or postvascular supernatant).

The supernatant volumes of distribution were corrected as follows:

15 Supernatant  $V_D = \{[^{125}\text{I}] V_D \times \% \text{ TCA precipitability} \} - \{[^3\text{H}] V_D\}$

The corrected supernatant  $V_D$  is a quantitative measure of protein distribution into the brain interstitium following transcytosis across the BBB. The subtraction of the  $V_D$  for [ $^3\text{H}$ ]-albumin was found to be necessary to correct for radioactive protein contained within the lumen of the brain vasculature that leaks from the vessels following homogenization and rupture of these vessels.

25

Clearance of [ $^{125}\text{I}$ ]-Histone and [ $^3\text{H}$ ]-Albumin Following a Single Intravenous Injection

An 0.5 ml aliquot of physiologic buffer containing 5  $\mu\text{Ci}$  of [ $^{125}\text{I}$ ]-histone and 50  $\mu\text{Ci}$  of [ $^3\text{H}$ ]-native albumin was rapidly injected into a femoral vein through a 27-gauge needle. At 0.25, 5, 30, 60, 120 and 180 minutes after the injection, the animal was quickly laparotomized and an 0.5 ml aliquot of arterial blood was removed from the descending aorta followed by decapitation of the animal and extirpation of the brain and nine other organs (heart, liver, spleen, testis, small intestine, skeletal muscle, fat, kidney and lung).

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The tissues were solubilized in Soluene-350 and analyzed with double isotope liquid scintillation counting. The blood [ $^3\text{H}$ ] and [ $^{125}\text{I}$ ] radioactivities were normalized to DPM/ml as a percent of injected dose, i.e.,  $A(t)$ , and these data were fit to the following biexponential function:

$$A(t) = A_1 e^{-K_1 t} + A_2 e^{-K_2 t}$$

using a derivative-free nonlinear regression analysis (PAR of BMDP, Biomedical Computer P Series Programs developed at UCLA Health Sciences Computing Facility). Because the standard error was roughly proportional to the means, the data were weighted using weight =  $1/[\text{clearance}]$ . The integral of the arterial radioactivity curve was determined from these data as follows:

$$\frac{A(t)dt}{t} = A_1 \frac{(1-e^{-K_1 t})}{K_1 t} + A_2 \frac{(1-e^{-K_2 t})}{K_2 t}$$

where  $t$  = time after injection. The volume of distribution of histone or albumin in brain and the nine other organs was determined from the ratio of DPM/gm tissue divided by integrated DPM/ml blood. The TCA precipitability of the serum [ $^3\text{H}$ ]-albumin was greater than 98% at all time points. However, there was a progressive decrease in the TCA precipitability of the [ $^{125}\text{I}$ ]-histone. Therefore, only arterial TCA precipitable [ $^{125}\text{I}$ ] counts were used in computation of the clearance of histone from blood or the organ volumes of distribution.

#### Uptake by Bovine Brain Microvessels

[ $^{125}\text{I}$ ]-histone was rapidly taken up by isolated bovine brain capillaries at  $37^\circ\text{C}$ , and approximately 25% of this uptake was resistant to mild acid wash and is presumed to represent internalized histone (see Fig. 3).

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Both the total binding and internalization were slowed by incubation at 4°C (see Fig. 3). The uptake of the [<sup>125</sup>I]-histone by isolated bovine brain capillaries was linear with respect to the amount of capillaries added to the incubation vessel throughout the range of 47-210 μg of capillary protein (data not shown). The binding of the [<sup>125</sup>I]-histone to the isolated brain microvessels was saturable with an ED<sub>50</sub> of approximately 300 μG/ML (14 μM) as shown by the data in Fig. 2. These saturation data were analyzed by Scatchard analysis to give the plot in Fig. 5. The dissociation constant (K<sub>D</sub>) = 15.2 ± 2.8 μM and the maximal binding or B<sub>max</sub> = 7.7 ± 1.0 nmol/mg protein. The binding of [<sup>125</sup>I]-histone to isolated bovine brain microvessels was inhibited by other polycationic proteins such as protamine or polylysine, but was minimally inhibited by cationized albumin and was not inhibited by cationized immunoglobulin G or native albumin (see Table 1).

Table 1. Competition for [<sup>125</sup>I]-Histone Binding to Isolated Bovine Brain Capillaries In Vitro

Medium	% Bound/mgp of [ <sup>125</sup> I]-Histone
Control	225 ± 10
2.5 mg/ml cationized immunoglobulin	232 ± 2
2.5 mg/ml native albumin	211 ± 9
0.5 mg/ml cationized albumin	171 ± 13 <sup>a</sup>
0.5 mg/ml histone	113 ± 4 <sup>b</sup>
0.5 mg/ml protamine	95 ± 5 <sup>b</sup>
2.5 mg/ml polylysine (59,000)	46 ± 1 <sup>b</sup>

Data are mean ± S.E. (n = 3).

a<sub>p</sub> < 0.01.

b<sub>p</sub> < 0.0005.

### Transport Through The Rat BBB In Vivo

The homogenate  $V_D$  for [ $^{125}\text{I}$ ]-histone increased with time and reached  $159 \pm 70 \mu\text{Lg}^{-1}$  by 10 minutes (see Table 2). Most of this [ $^{125}\text{I}$ ]-histone taken up by brain, however, was sequestered in the vascular compartment, as the postvascular supernatant  $V_D$  of [ $^{125}\text{I}$ ]-histone at 10 minutes was  $12 \pm 5 \mu\text{Lg}^{-1}$ , which represents 8% of the total uptake. The [ $^{125}\text{I}$ ]-histone in the supernatant that was TCA precipitable was  $90 \pm 3\%$  by 10 minutes of perfusion. The homogenate  $V_D$  for [ $^{125}\text{I}$ ]-histone was nearly ten-fold greater than the homogenate  $V_D$  for [ $^3\text{H}$ ]-albumin (see Table 2). By definition, the supernatant  $V_D$  for [ $^3\text{H}$ ]-albumin = 0. The pellet  $V_D$  for [ $^3\text{H}$ ]-albumin was  $0.76 \pm 0.20 \mu\text{Lg}^{-1}$  at 10 minutes of perfusion, which is >100-fold less than the pellet  $V_D$  for [ $^{125}\text{I}$ ]-histone (see Table 2).

Table 2. Volume of Distribution ( $V_D$ ) of [ $^{125}\text{I}$ ]-Histone or [ $^3\text{H}$ ]-Albumin after 1 to 10 Minute Perfusions in Rat Brain In Vivo

Protein	Brain Fraction	Time (min)			
		1	2.5	5	10
[ $^{125}\text{I}$ ]-histone	Homogenate	5.1 $\pm$ 3.5	20 $\pm$ 10	64 $\pm$ 13	159 $\pm$ 70
	Supernatant	1.6 $\pm$ 0.5	1.4 $\pm$ 0.1	12 $\pm$ 4	12 $\pm$ 5
	Pellet	0.9 $\pm$ 0.5	10 $\pm$ 8	39 $\pm$ 9	118 $\pm$ 59
[ $^3\text{H}$ ]-albumin	Homogenate	2.2 $\pm$ 0.7	2.8 $\pm$ 0.4	18 $\pm$ 7	17 $\pm$ 7

Data are mean  $\pm$  S.E. (n = 3-7). Reported as  $V_D$ ,  $\mu\text{L/g}$ .

### Clearance of [ $^{125}\text{I}$ ]-Histone and [ $^3\text{H}$ ]-Albumin from Blood following a Single Intravenous Injection

The decay is plasma [ $^{125}\text{I}$ ]-histone that was TCA precipitable and the decay in plasma [ $^3\text{H}$ ]-albumin following a single intravenous injection is shown in Fig. 6. The [ $^3\text{H}$ ]-albumin data could not be fit to a

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biexponential function, but did fit a monoexponential function. The [ $^{125}\text{I}$ ]-histone blood data could not be fit to either a monoexponential or a triexponential function but did fit to a bioexponential function, and the intercepts and slopes of the two exponential decays are given in Fig. 6.

Following rapid clearance from blood, [ $^{125}\text{I}$ ]-histone was cleared monoexponentially with a half-time of  $2.0 \pm 0.5$  hours, and this value was about 40% of the half-time for [ $^3\text{H}$ ]-albumin clearance,  $4.8 \pm 1.8$  hours (see Fig. 6). The 60-minute brain  $V_D$  of [ $^{125}\text{I}$ ]-histone and the ratios of the 60-minute [ $^{125}\text{I}$ ]-histone  $V_D$ /[ $^3\text{H}$ ]-albumin  $V_D$  for brain and nine other organs are shown in Table 3. The 60-minute  $V_D$  data are shown because, with the exception of testis and small intestine, brain and the other organs reached maximal organ distribution by 60 minutes. The 180-minute  $V_D$  was 41% and 53% higher than the 60-minute  $V_D$  for testis and small intestines, respectively. The 180-minute  $V_D$  was 40% lower than the 60-minute  $V_D$  for brain, lung, and spleen. The  $V_D$  was essentially unchanged at 60 or 180 minutes for liver, heart, kidney, muscle, or fat.

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Table 3. Volumes of Distribution ( $V_D$ ) of Histone and Albumin at 60 Minutes Following Intravenous Injection

5	Organ	Ratio of		
		Histone $V_D$ (ml/g)	Albumin $V_D$ (ml/g)	$V_D$ -Histone/ $V_D$ -Albumin
	Kidney	7.9±0.5	0.36±0.02	22.3±1.4
	Muscle	0.71±0.01	0.036±0.002	19.6±0.2
10	Spleen	6.0±0.8	0.35±0.03	17.5±2.4
	Small Intestine	2.0±0.7	0.11±0.01	17.4±6.1
	Liver	4.6±0.2	0.34±0.02	13.7±0.7
	Lung	5.1±0.3	0.38±0.03	13.3±0.7
15	Brain	0.17±0.02	0.017±0.002	10.0±1.2
	Testis	0.68±0.04	0.086±0.011	7.9±0.5
	Fat	0.32±0.02	0.048±0.006	6.7±0.5
	Heart	0.94±0.10	0.21±0.03	4.5±0.5
20	$V_D$ shown are data obtained 60 minutes after single intravenous injection. Data are mean ± S.E. (n = 3).			

This example demonstrates that [ $^{125}\text{I}$ ]-histone binds both the luminal and antiluminal sides of the brain capillary. The in vivo perfusion studies in Table 2 showing the very high microvascular pellet  $V_D$  for [ $^{125}\text{I}$ ]-histone relative to [ $^3\text{H}$ ]-albumin, demonstrate that [ $^{125}\text{I}$ ]-histone is bound by the luminal membrane of the brain capillary. Conversely, there must also be binding to the antiluminal membrane to explain the rapid binding within 5 seconds of incubation with isolated bovine brain microvessels (see Fig. 3).

The binding of the [ $^{125}\text{I}$ ]-histone to brain microvessels is temperature-dependent (Fig. 3) and is saturable (Fig. 2). The saturation  $\text{ED}_{50}$  of 14  $\mu\text{M}$  histone indicates that the capacity of the histone

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uptake system is very high. For example, the saturation ED<sub>50</sub> for cationized albumin was 0.05 mg/ml (0.7  $\mu$ M) (Kumagai et al.: Absorptive-mediated endocytosis of cationized albumin and a  $\beta$ -endorphin-cationized albumin chimeric peptide by isolated brain capillaries. Model system of blood-brain barrier transport. J. Biol. Chem. 262: 15214-15219, 1987), and for cationized immunoglobulin was 1 mg/ml (6  $\mu$ M) (Triguero et al.: Cationization of immunoglobulin G (IgG) as a new strategy for enhanced IgG delivery through the blood-brain barrier. Clin. Res. 37: 140A). The B<sub>max</sub> for histone of  $7.7 \pm 1.0$  nmol/mg protein is approximately five-fold the B<sub>max</sub> for cationized immunoglobulin G and is approximately ninety-fold greater than the B<sub>max</sub> for binding of cationized albumin. The differing B<sub>max</sub> values for the various polycationic proteins suggests that these molecules bind to different groups of negative charges on the brain capillary endothelial membrane. For example, cationized albumin only weakly inhibits histone uptake, whereas cationized immunoglobulin has no effect, as polylysine (see Table 1).

Histone undergoes absorptive-mediated endocytosis into brain capillary endothelial cytoplasm following its binding to the surface of the capillary, as demonstrated by the resistance to mild acid wash (Fig. 3). Moreover, the data in Table 2 show that approximately 8% of the total histone bound and endocytosed by the brain capillary undergoes exocytosis into the brain interstitium in vivo, which completes an overall pathway of transcytosis through the capillary endothelium.

All of the references set forth in the preceding examples are hereby incorporated by reference.

Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the within disclosures are exemplary only and that various other alternatives, adaptations



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and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein, but is only limited by the following

5 claims.

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What is claimed is:

1. A chimeric peptide adapted for delivering a neuropharmaceutical agent into the brain by transcytosis through the blood-brain barrier, said chimeric peptide comprising a transportable peptide capable of crossing  
5 the blood-brain barrier by transcytosis conjugated with said neuropharmaceutical agent, wherein said transportable peptide is histone.

2. A chimeric peptide according to claim 1 wherein said histone is isolated from a human source.

3. A chimeric peptide according to claim 1 wherein said histone is selected from a class I-V type of histone.

4. A chimeric peptide according to claim 2 wherein said histone is selected from a class I-V type of histone.

5. A chimeric peptide according to claim 1 wherein said neuropharmaceutical agent is a hydrophilic peptide.

6. A chimeric peptide according to claim 5 wherein said neuropharmaceutical agent is selected from the group consisting of somatostatin, thyrotropin releasing hormone, vasopressin, alpha interferon, endorphin,  
5 muramyl dipeptide and L-methionyl(sulfone)-L-glytamyl-L-histidyl-L-phenylalanyl-D-lysyl-L-phenylalanine.

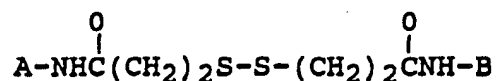
7. A chimeric peptide according to claim 1 wherein said transportable peptide and neuropharmaceutical agent are conjugated via a conjugation agent.

8. A chimeric peptide according to claim 1 wherein

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said conjugation agent is capable of conjugating the transportable peptide to said neuropharmaceutical agent by peptide thiolation or lysine coupling via glutaraldehyde.

9. A chimeric peptide according to claim 1 having the formula



5 wherein A is a neuropharmaceutical agent and B is histone.

10. A chimeric peptide according to claim 9 wherein A is selected from the group consisting of somatostatin, thyrotropin releasing hormone, vasopressin, alpha interferon, endorphin, muramyl dipeptide and L-methionyl(sulfone)-L-glytamyl-L-histidyl-L-phenylalanyl-D-lysyl-L-phenylalanine.

11. A composition comprising a chimeric peptide according to claim 1 and a pharmaceutically acceptable carrier for said chimeric peptide.

12. A composition according to claim 11 wherein said pharmaceutically acceptable carrier is sterile saline.

13. A method for delivering a neuropharmaceutical agent into the brain of an animal by transcytosis through the blood-brain barrier comprising the step of introducing a chimeric peptide according to claim 1 into the bloodstream of said animal in a sufficient amount to provide transport of said chimeric peptide across said blood-brain barrier.

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14. A method according to claim 13 wherein said chimeric peptide is introduced intranasally into the subject's bloodstream.

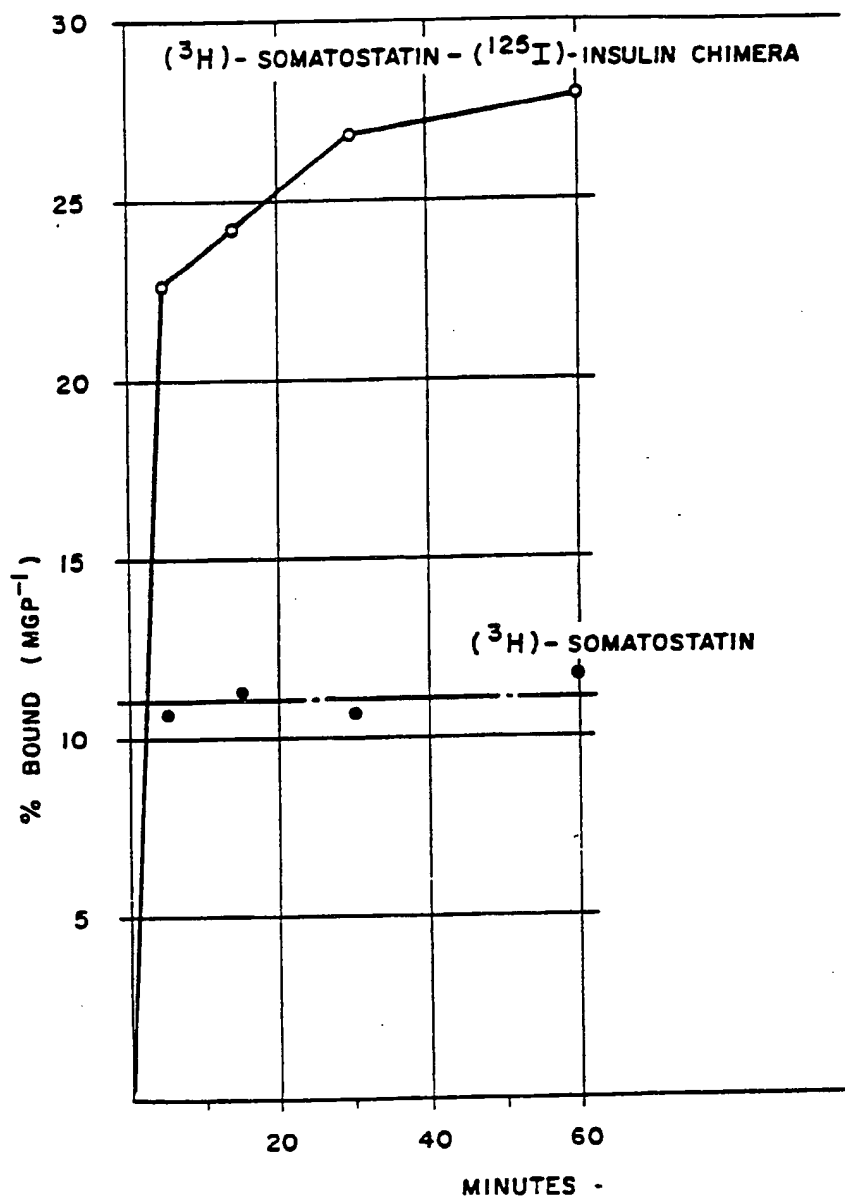
15. In a method for introducing a hydrophilic neuropeptide into the brain across the blood-brain barrier, wherein the improvement comprises increasing the rate at which said neuropeptide crosses the blood-  
5 brain barrier by conjugating said neuropeptide with histone.

16. The improved method according to claim 15 wherein said histone is isolated from a human source.

17. The improved method according to claim 15 wherein said histone is selected from a class I-V type of histone.

18. The improved method according to claim 16 wherein said histone is selected from a class I-V type of histone.

1/5

*Fig. 1.*

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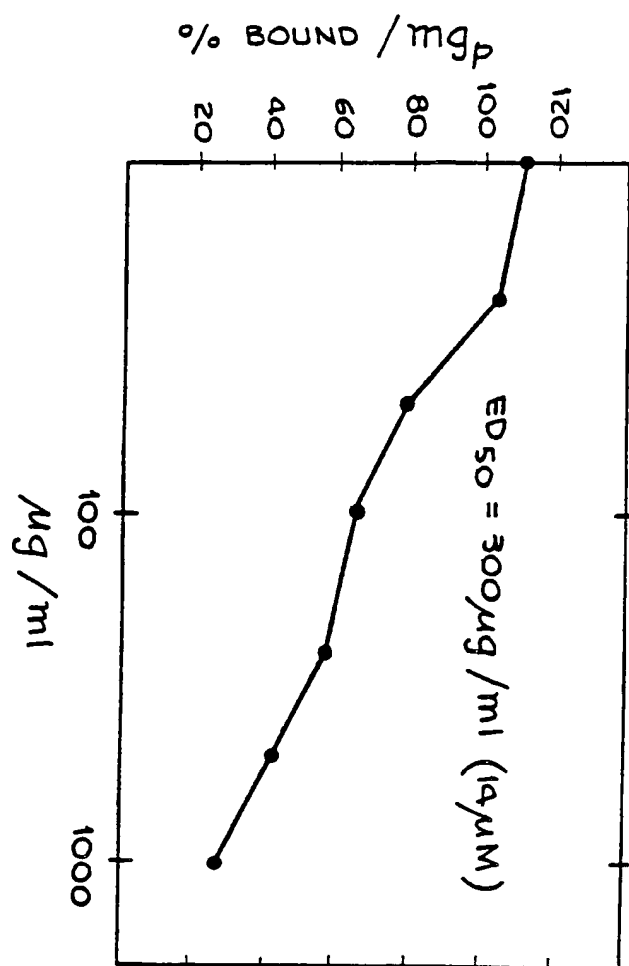


Fig. 2.

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Fig. 3a

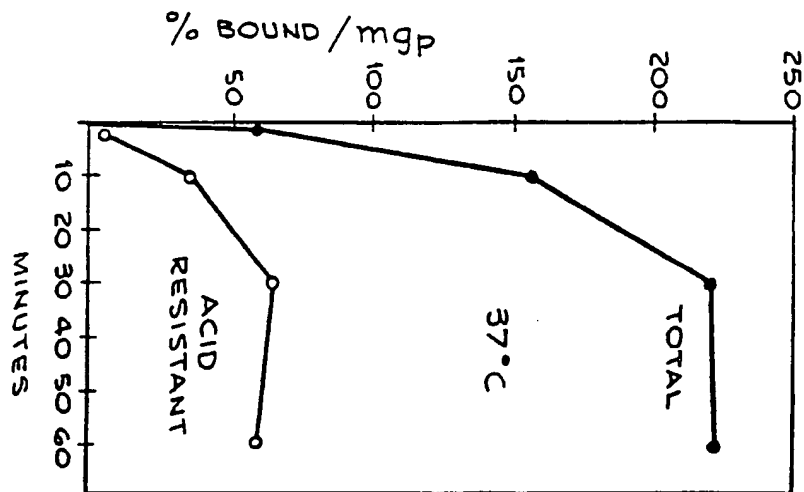
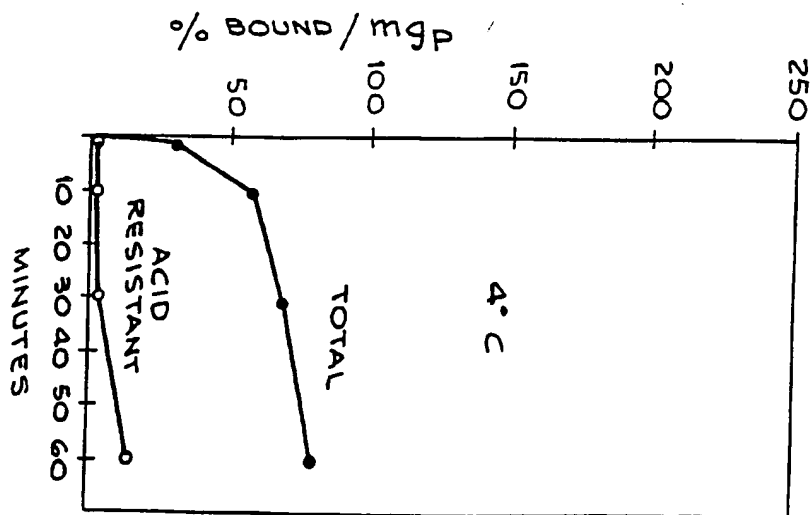
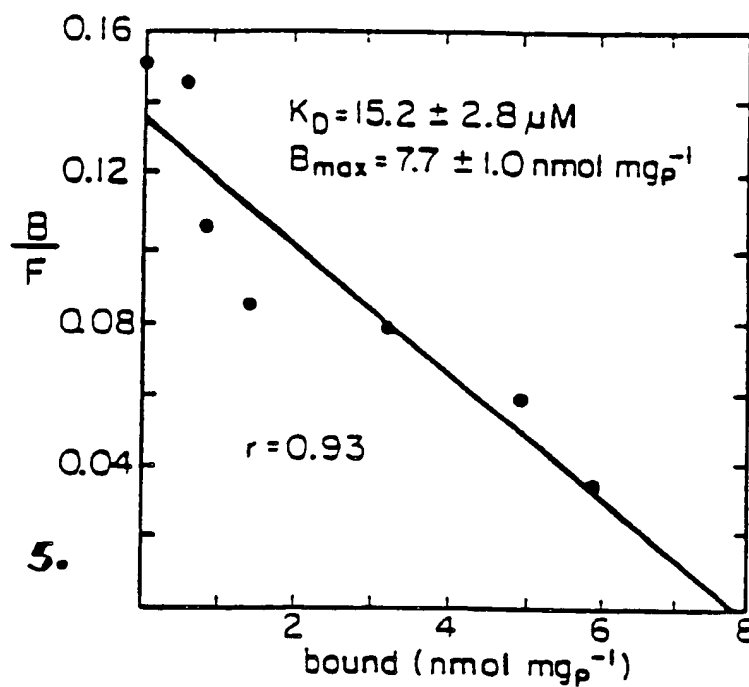
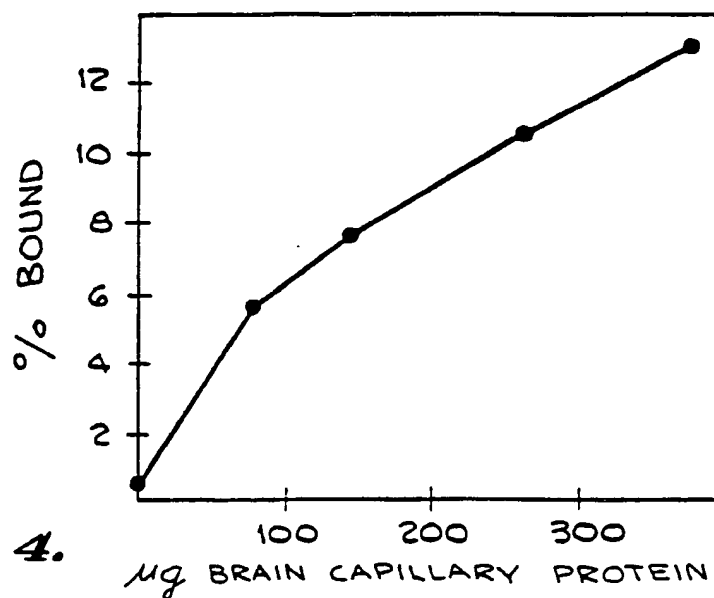


Fig. 3b



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*Fig. 5.**Fig. 4.*



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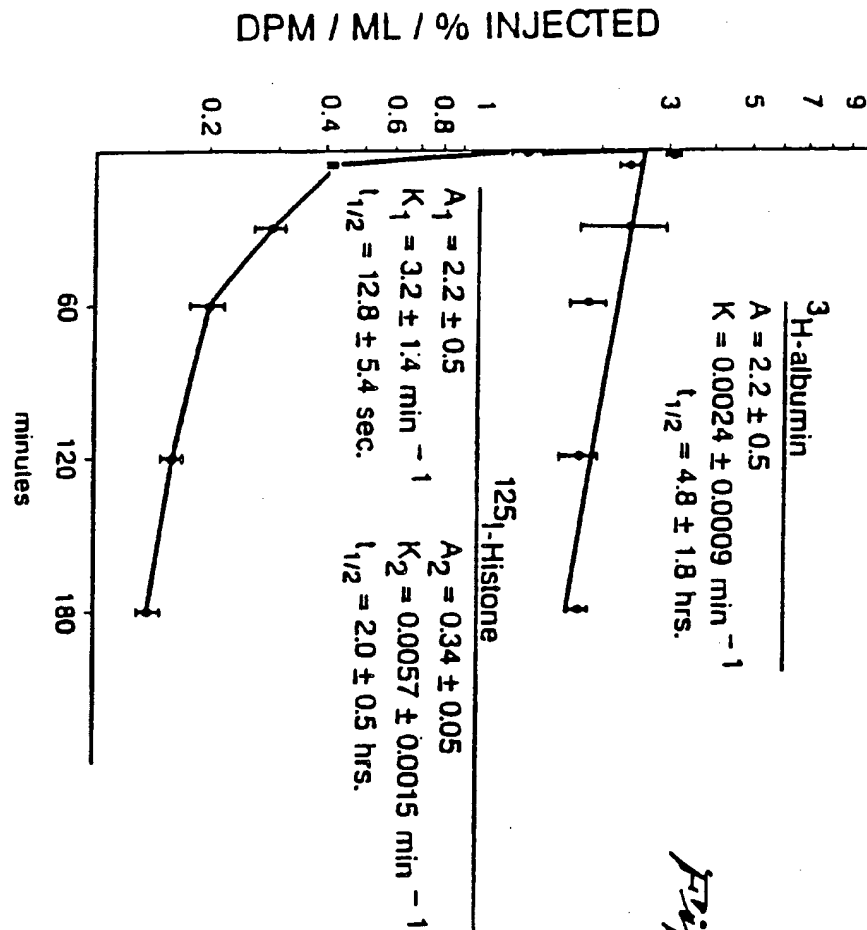



Fig. 6.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/01589

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC <sup>4</sup> : A 61 K 37/02, 37/26, 45/02, //(A 61 K 37/26, 37:02), (A 61 K 45/02, 37:02)		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
IPC <sup>4</sup>	A 61 K, C 07 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched *		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT *</b>		
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	WO, A, 88/00834 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 11 February 1988 see the whole document --	1-12,15- 18
X	Chemical Abstracts, volume 107, no. 12, 21 September 1987, (Columbus, Ohio, US), W.M. Pardridge et al.: "Chimeric peptides as a vehicle for peptide pharmaceutical delivery through the blood-brain barrier", see page 379, abstract 102538b, & Biochem. Biophys. Res. Commun. 1987, 146(1), 307-13 -----	1-12,15- 18
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
14th August 1989	19. 09. 89	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	 <b>T.K. WILLIS</b>	

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers XX..... because they relate to subject matter not required to be searched by this Authority, namely:

xx 13,14

pls. see Rule 39.1 (IV) - PCT

Methods for treatment of the human or animal body by surgery or therapy as well as diagnostic methods.

2. ☐ Claim numbers..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 8901589

SA 28164

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on 06/09/89  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8800834	11-02-88	US-A- 4801575	31-01-89
		EP-A- 0276278	03-08-88
		JP-T- 1500901	30-03-89
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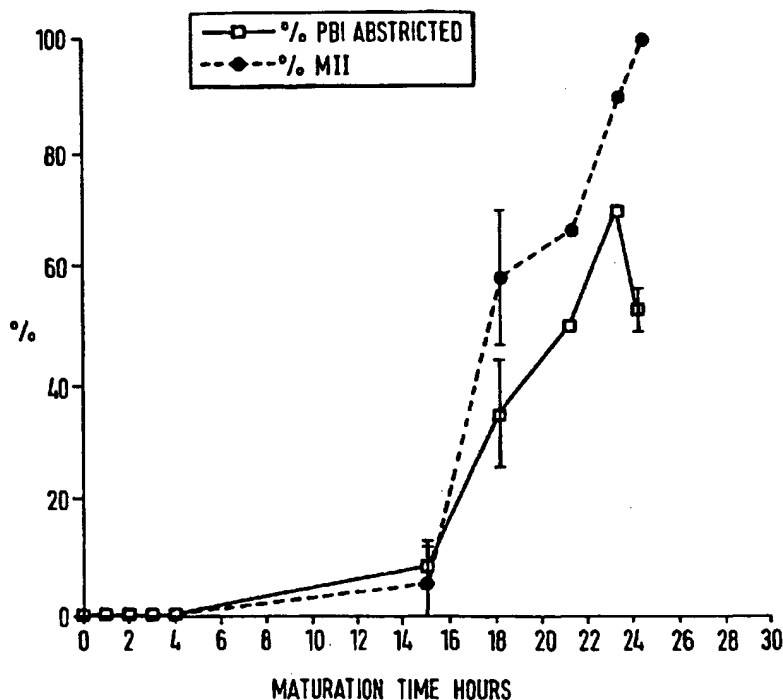
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A01K 67/027, A61K 35/54</b>		A1	(11) International Publication Number: <b>WO 97/07668</b>
			(43) International Publication Date: 6 March 1997 (06.03.97)
(21) International Application Number: PCT/GB96/02098 (22) International Filing Date: 30 August 1996 (30.08.96) (30) Priority Data: 9517779.6                      31 August 1995 (31.08.95)                      GB (71) Applicant (for all designated States except US): ROSLIN INSTITUTE (EDINBURGH) [GB/GB]; Roslin, Midlothian EH25 9PS (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): CAMPBELL, Keith, Henry, Stockman [GB/GB]; Roslin Institute (Edinburgh), Roslin, Midlothian EH25 9PS (GB). WILMUT, Ian [GB/GB]; Roslin Institute (Edinburgh), Roslin, Midlothian EH25 9PS (GB). (74) Agents: CHAPMAN, Paul, William et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB).			(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  Published With international search report.

(54) Title: UNACTIVATED OOCYTES AS CYTOPLAST RECIPIENTS FOR NUCLEAR TRANSFER

## (57) Abstract

A method of reconstituting an animal embryo involves transferring a diploid nucleus into an oocyte which is arrested in the metaphase of the second meiotic division. The oocyte is not activated at the time of transfer, so that the donor nucleus is kept exposed to the recipient cytoplasm for a period of time. The diploid nucleus can be donated by a cell in either the G0 or G1 phase of the cell cycle at the time of transfer. Subsequently, the reconstituted embryo is activated. Correct ploidy is maintained during activation, for example, by incubating the reconstituted embryo in the presence of a microtubule inhibitor such as nocodazole. The reconstituted embryo may then give rise to one or more live animal births. The invention is useful in the production of transgenic animals as well as non-transgenics of high genetic merit.



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UNACTIVATED OOCYTES AS CYTOPLAST RECIPIENTS  
FOR NUCLEAR TRANSFER

5 This invention relates to the generation of animals including but not being limited to genetically selected and/or modified animals, and to cells useful in their generation.

10 The reconstruction of mammalian embryos by the transfer of a donor nucleus to an enucleated oocyte or one cell zygote allows the production of genetically identical individuals. This has clear advantages for both research (i.e. as biological controls) and also in commercial applications (i.e. multiplication of genetically valuable  
15 livestock, uniformity of meat products, animal management).

Embryo reconstruction by nuclear transfer was first proposed (Spemann, *Embryonic Development and Induction*  
20 210-211 Hofner Publishing Co., New York (1938)) in order to answer the question of nuclear equivalence or 'do nuclei change during development?'. By transferring nuclei from increasingly advanced embryonic stages these experiments were designed to determine at which point  
25 nuclei became restricted in their developmental potential. Due to technical limitations and the unfortunate death of Spemann these studies were not completed until 1952, when it was demonstrated in the frog that certain nuclei could direct development to a  
30 sexually mature adult (Briggs and King, *Proc. Natl. Acad. Sci. USA* 38 455-461 (1952)). Their findings led to the current concept that equivalent totipotent nuclei from a single individual could, when transferred to an enucleated egg, give rise to "genetically identical"



5 individuals. In the true sense of the meaning these individuals would not be clones as unknown cytoplasmic contributions in each may vary and also the absence of any chromosomal rearrangements would have to be demonstrated.

Since the demonstration of embryo cloning in amphibians, similar techniques have been applied to mammalian species. These techniques fall into two categories:

10 1) transfer of a donor nucleus to a matured metaphase II oocyte which has had its chromosomal DNA removed and

2) transfer of a donor nucleus to a fertilised one cell zygote which has had both pronuclei removed. In ungulates the former procedure has become the method of

15 choice as no development has been reported using the latter other than when pronuclei are exchanged.

Transfer of the donor nucleus into the oocyte cytoplasm is generally achieved by inducing cell fusion. In

20 ungulates fusion is induced by application of a DC electrical pulse across the contact/fusion plane of the couplet. The same pulse which induces cell fusion also activates the recipient oocyte. Following embryo reconstruction further development is dependent on a

25 large number of factors including the ability of the nucleus to direct development i.e. totipotency, developmental competence of the recipient cytoplasm (i.e. oocyte maturation), oocyte activation, embryo culture (reviewed Campbell and Wilmut in *Vth World Congress on*

30 *Genetics as Applied to Livestock* 20 180-187 (1994)).

In addition to the above we have shown that maintenance of correct ploidy during the first cell cycle of the reconstructed embryo is of major importance (Campbell

et al., *Biol. Reprod.* 49 933-942 (1993); Campbell et al., *Biol. Reprod.* 50 1385-1393 (1994)). During a single cell cycle all genomic DNA must be replicated once and only once prior to mitosis. If any of the DNA either fails to replicate or is replicated more than once then the ploidy of that nucleus at the time of mitosis will be incorrect. The mechanisms by which replication is restricted to a single round during each cell cycle are unclear, however, several lines of evidence have implicated that maintenance of an intact nuclear membrane is crucial to this control. The morphological events which occur in the donor nucleus after transfer into an enucleated metaphase II oocyte have been studied in a number of species including mouse (Czolowska et al., *J. Cell Sci.* 69 19-34 (1984)), rabbit (Collas and Robl, *Biol. Reprod.* 45 455-465 (1991)), pig (Prather et al., *J. Exp. Zool.* 225 355-358 (1990)), cow (Kanka et al., *Mol. Reprod. Dev.* 29 110-116 (1991)). Immediately upon fusion the donor nuclear envelope breaks down (NEBD), and the chromosomes prematurely condense (PCC). These effects are catalysed by a cytoplasmic activity termed maturation/mitosis/meiosis promoting factor (MPF). This activity is found in all mitotic and meiotic cells reaching a maximal activity at metaphase. Matured mammalian oocytes are arrested at metaphase of the 2nd meiotic division (metaphase II) and have high MPF activity. Upon fertilisation or activation MPF activity declines, the second meiotic division is completed and the second polar body extruded, the chromatin then decondenses and pronuclear formation occurs. In nuclear transfer embryos reconstructed when MPF levels are high NEBD and PCC occur; these events are followed, when MPF activity declines, by chromatin decondensation and nuclear reformation and subsequent DNA replication. In

reconstructed embryos correct ploidy can be maintained in one of two ways; firstly by transferring nuclei at a defined cell cycle stage, e.g. diploid nuclei of cells in G1, into metaphase II oocytes at the time of activation; or secondly by activating the recipient oocyte and transferring the donor nucleus after the disappearance of MPF activity. In sheep this latter approach has yielded an increase in the frequency of development to the blastocyst stage from 21% to 55% of reconstructed embryos when using blastomeres from 16 cell embryos as nuclear donors (Campbell et al., *Biol. Reprod.* 50 1385-1393 (1994)).

These improvements in the frequency of development of reconstructed embryos have as yet not addressed the question of nuclear reprogramming. During development certain genes become "imprinted" i.e. are altered such that they are no longer transcribed. Studies on imprinting have shown that this "imprinting" is removed during germ cell formation (i.e. reprogramming). One possibility is that this reprogramming is affected by exposure of the chromatin to cytoplasmic factors which are present in cells undergoing meiosis. This raises the question of how we may mimic this situation during the reconstruction of embryos by nuclear transfer in order to reprogram the developmental clock of the donor nucleus.

It has now been found that nuclear transfer into an oocyte arrested in metaphase II can give rise to a viable embryo if normal ploidy (i.e. diploidy) is maintained and if the embryo is not activated at the time of nuclear transfer. The delay in activation allows the nucleus to remain exposed to the recipient cytoplasm.

According to a first aspect of the present invention there is provided a method of reconstituting an animal embryo, the method comprising transferring a diploid nucleus into an oocyte which is arrested in the metaphase of the second meiotic division without concomitantly activating the oocyte, keeping the nucleus exposed to the cytoplasm of the recipient for a period of time sufficient for the reconstituted embryo to become capable of giving rise to a live birth and subsequently activating the reconstituted embryo while maintaining correct ploidy. At this stage, the reconstituted embryo is a single cell.

In principle, the invention is applicable to all animals, including birds such as domestic fowl, amphibian species and fish species. In practice, however, it will be to non-human animals, especially non-human mammals, particularly placental mammals, that the greatest commercially useful applicability is presently envisaged. It is with ungulates, particularly economically important ungulates such as cattle, sheep, goats, water buffalo, camels and pigs that the invention is likely to be most useful, both as a means for cloning animals and as a means for generating transgenic animals. It should also be noted that the invention is also likely to be applicable to other economically important animal species such as, for example, horses, llamas or rodents, e.g. rats or mice, or rabbits.

The invention is equally applicable in the production of transgenic, as well as non-transgenic animals. Transgenic animals may be produced from genetically altered donor cells. The overall procedure has a number of advantages over conventional procedures for the production of

transgenic (i.e. genetically modified) animals which may be summarised as follows:

- (1) fewer recipients will be required;
- 5       (2) multiple syngeneic founders may be generated using clonal donor cells;
- (3) subtle genetic alteration by gene targeting is permitted;
- 10       (4) all animals produced from embryos prepared by the invention should transmit the relevant genetic modification through the germ line as each animal is derived from a single nucleus; in contrast, production of transgenic animals by pronuclear injection or chimerism after  
15       inclusion of modified stem cell populations by blastocyst injection produces a proportion of mosaic animals in which all cells do not contain the modification and may not transmit the modification through the germ line; and  
20       (5) cells can be selected for the site of genetic modification (e.g. integration) prior to the generation of the whole animal.

It should be noted that the term "transgenic", in  
25       relation to animals, should not be taken to be limited to referring to animals containing in their germ line one or more genes from another species, although many transgenic animals will contain such a gene or genes. Rather, the term refers more broadly to any animal whose germ line  
30       has been the subject of technical intervention by recombinant DNA technology. So, for example, an animal in whose germ line an endogenous gene has been deleted, duplicated, activated or modified is a transgenic animal for the purposes of this invention as much as an animal

to whose germ line an exogenous DNA sequence has been added.

5 In embodiments of the invention in which the animal is transgenic, the donor nucleus is genetically modified. The donor nucleus may contain one or more transgenes and the genetic modification may take place prior to nuclear transfer and embryo reconstitution. Although micro-injection, analogous to injection into the male or female  
10 pronucleus of a zygote, may be used as a method of genetic modification, the invention is not limited to that methodology: mass transformation or transfection techniques can also be used e.g. electroporation, viral transfection or lipofection.

15 In the method of the invention described above, a diploid nucleus is transferred from a donor into the enucleated recipient oocyte. Donors which are diploid at the time of transfer are necessary in order to maintain the correct ploidy of the reconstituted embryo; therefore  
20 donors may be either in the G1 phase or preferably, as is the subject of our co-pending PCT patent application No. PCT/GB96/02099 filed today (claiming priority from GB 9517780.4), in the G0 phase of the cell cycle.

25 The mitotic cell cycle has four distinct phases, G, S, G2 and M. The beginning event in the cell cycle, called start, takes place in the G1 phase and has a unique function. The decision or commitment to undergo another  
30 cell cycle is made at start. Once a cell has passed through start, it passes through the remainder of the G1 phase, which is the pre-DNA synthesis phase. The second stage, the S phase, is when DNA synthesis takes place. This is followed by the G2 phase, which is the period

between DNA synthesis and mitosis. Mitosis itself occurs at the M phase. Quiescent cells (which include cells in which quiescence has been induced as well as those cells which are naturally quiescent, such as certain fully differentiated cells) are generally regarded as not being in any of these four phases of the cycle; they are usually described as being in a G0 state, so as to indicate that they would not normally progress through the cycle. The nuclei of quiescent G0 cells, like the nuclei of G1 cells, have a diploid DNA content; both of such diploid nuclei can be used in the present invention.

Subject to the above, it is believed that there is no significant limitation on the cells that can be used in nuclear donors: fully or partially differentiated cells or undifferentiated cells can be used as can cells which are cultured *in vitro* or abstracted *ex vivo*. The only limitation is that the donor cells have normal DNA content and be karyotypically normal. A preferred source of cells is disclosed in our co-pending PCT patent application No. PCT/GB95/02095, published as WO 96/07732. It is believed that all such normal cells contain all of the genetic information required for the production of an adult animal. The present invention allows this information to be provided to the developing embryo by altering chromatin structure such that the genetic material can re-direct development.

Recipient cells useful in the invention are enucleated oocytes which are arrested in the metaphase of the second meiotic division. In most vertebrates, oocyte maturation proceeds *in vivo* to this fairly late stage of the egg maturation process and then arrests. At ovulation, the arrested oocyte is released from the ovary (and, if

fertilisation occurs, the oocyte is naturally stimulated to complete meiosis). In the practice of the invention, oocytes can be matured either *in vitro* or *in vivo* and are collected on appearance of the 1st polar body or as soon as possible after ovulation, respectively.

It is preferred that the recipient be enucleate. While it has been generally assumed that enucleation of recipient oocytes in nuclear transfer procedures is essential, there is no published experimental confirmation of this judgement. The original procedure described for ungulates involved splitting the cell into two halves, one of which was likely to be enucleated (Willadsen *Nature* 320 (6) 63-65 (1986)). This procedure has the disadvantage that the other unknown half will still have the metaphase apparatus and that the reduction in volume of the cytoplasm is believed to accelerate the pattern of differentiation of the new embryo (Eviskov *et al.*, *Development* 109 322-328 (1990)).

More recently, different procedures have been used in attempts to remove the chromosomes with a minimum of cytoplasm. Aspiration of the first polar body and neighbouring cytoplasm was found to remove the metaphase II apparatus in 67% of sheep oocytes (Smith & Wilmut *Biol. Reprod.* 40 1027-1035 (1989)). Only with the use of DNA-specific fluorochrome (Hoechst 33342) was a method provided by which enucleation would be guaranteed with the minimum reduction in cytoplasmic volume (Tsunoda *et al.*, *J. Reprod. Fertil.* 82 173 (1988)). In livestock species, this is probably the method of routine use at present (Prather & First *J. Reprod. Fertil. Suppl.* 41 125 (1990), Westhusin *et al.*, *Biol. Reprod. (Suppl.)* 42 176 (1990)).



There have been very few reports of non-invasive approaches to enucleation in mammals, whereas in amphibians, irradiation with ultraviolet light is used as a routine procedure (Gurdon *Q. J. Microsc. Soc.* 101 299-311 (1960)). There are no detailed reports of the use of this approach in mammals, although during the use of DNA-specific fluorochrome it was noted that exposure of mouse oocytes to ultraviolet light for more than 30 seconds reduced the developmental potential of the cell (Tsunoda *et al.*, *J. Reprod. Fertil.* 82 173 (1988)).

As described above enucleation may be achieved physically, by actual removal of the nucleus, pro-nuclei or metaphase plate (depending on the recipient cell), or functionally, such as by the application of ultraviolet radiation or another enucleating influence.

After enucleation, the donor nucleus is introduced either by fusion to donor cells under conditions which do not induce oocyte activation or by injection under non-activating conditions. In order to maintain the correct ploidy of the reconstructed embryo the donor nucleus must be diploid (i.e. in the G0 or G1 phase of the cell cycle) at the time of fusion.

Once suitable donor and recipient cells have been prepared, it is necessary for the nucleus of the former to be transferred to the latter. Most conveniently, nuclear transfer is effected by fusion. Activation should not take place at the time of fusion.

Three established methods which have been used to induce fusion are:

- (1) exposure of cells to fusion-promoting chemicals, such as polyethylene glycol;
- (2) the use of inactivated virus, such as Sendai virus; and
- 5 (3) the use of electrical stimulation.

Exposure of cells to fusion-promoting chemicals such as polyethylene glycol or other glycols is a routine procedure for the fusion of somatic cells, but it has not  
10 been widely used with embryos. As polyethylene glycol is toxic it is necessary to expose the cells for a minimum period and the need to be able to remove the chemical quickly may necessitate the removal of the zona pellucida (Kanka et al., *Mol. Reprod. Dev.* 29 110-116 (1991)). In  
15 experiments with mouse embryos, inactivated Sendai virus provides an efficient means for the fusion of cells from cleavage-stage embryos (Graham *Wistar Inst. Symp. Monogr.* 9 19 (1969)), with the additional experimental advantage that activation is not induced. In ungulates, fusion is  
20 commonly achieved by the same electrical stimulation that is used to induce parthogenetic activation (Willadsen *Nature* 320 (6) 63-65 (1986), Prather et al., *Biol. Reprod.* 37 859-866 (1987)). In these species, Sendai virus induces fusion in a proportion of cases, but is not  
25 sufficiently reliable for routine application (Willadsen *Nature* 320 (6) 63-65 (1986)).

While cell-cell fusion is a preferred method of effecting nuclear transfer, it is not the only method that can be  
30 used. Other suitable techniques include microinjection (Ritchie and Campbell, *J. Reproduction and Fertility Abstract Series No. 15*, p60).

In a preferred embodiment of the invention, fusion of the oocyte karyoplast couplet is accomplished in the absence of activation by electropulsing in 0.3M mannitol solution or 0.27M sucrose solution; alternatively the nucleus may  
5 be introduced by injection in a calcium free medium. The age of the oocytes at the time of fusion/injection and the absence of calcium ions from the fusion/injection medium prevent activation of the recipient oocyte.

10 In practice, it is best to enucleate and conduct the transfer s soon as possible after the oocyte reaches metaphase II. The time that this will be post onset of maturation (*in vitro*) or hormone treatment (*in vivo*) will depend on the species. For cattle or sheep, nuclear  
15 transfer should preferably take place within 24 hours; for pigs, within 48 hours; mice, within 12 hours; and rabbits within 20-24 hours. although transfer can take place later, it becomes progressively more difficult to achieve as the oocyte ages. High MPF activity is  
20 desirable.

Subsequently, the fused reconstructed embryo, which is generally returned to the maturation medium, is maintained without being activated so that the donor  
25 nucleus is exposed to the recipient cytoplasm for a period of time sufficient to allow the reconstructed embryo to become capable, eventually, of giving rise to a live birth (preferably of a fertile offspring).

30 The optimum period of time before activation varies from species to species and can readily be determined by experimentation. For cattle, a period of from 6 to 20 hours is appropriate. The time period should probably not be less than that which will allow chromosome

formation, and it should not be so long either that the couplet activates spontaneously or, in extreme cases that it dies.

5 When it is time for activation, any conventional or other  
suitable activation protocol can be used. Recent  
experiments have shown that the requirements for  
parthogenetic activation are more complicated than had  
10 been imagined. It had been assumed that activation is an  
all-or-none phenomenon and that the large number of  
treatments able to induce formation of a pronucleus were  
all causing "activation". However, exposure of rabbit  
oocytes to repeated electrical pulses revealed that only  
15 selection of an appropriate series of pulses and control  
of the  $\text{Ca}^{2+}$  was able to promote development of diploidized  
oocytes to mid-gestation (Ozil *Development* 109 117-127  
(1990)). During fertilization there are repeated,  
transient increases in intracellular calcium  
20 concentration (Cutbertson & Cobbold *Nature* 316 541-542  
(1985)) and electrical pulses are believed to cause  
analogous increases in calcium concentration. There is  
evidence that the pattern of calcium transients varies  
with species and it can be anticipated that the optimal  
25 pattern of electrical pulses will vary in a similar  
manner. The interval between pulses in the rabbit is  
approximately 4 minutes (Ozil *Development* 109 117-127  
(1990)), and in the mouse 10 to 20 minutes (Cutbertson &  
Cobbold *Nature* 316 541-542 (1985)), while there are  
30 preliminary observations in the cow that the interval is  
approximately 20 to 30 minutes (Robl et al., in *Symposium  
on Cloning Mammals by Nuclear Transplantation* (Seidel  
ed.), Colorado State University, 24-27 (1992)). In most  
published experiments activation was induced with a  
single electrical pulse, but new observations suggest

that the proportion of reconstituted embryos that develop is increased by exposure to several pulses (Collas & Robl *Biol. Reprod.* 43 877-884 (1990)). In any individual case, routine adjustments may be made to optimise the number of pulses, the field strength and duration of the pulses and the calcium concentration of the medium.

In the practice of the invention, correct ploidy must be maintained during activation. It is desirable to inhibit or stabilise microtubule polymerisation in order to prevent the production of multiple pronuclei, thereby to maintain correct ploidy. This can be achieved by the application of a microtubule inhibitor such as nocodazole at an effective concentration (such as about 5µg/ml). Colchicine and colcemid are other microtubule inhibitors. Alternatively, a microtubule stabiliser, such as, for example, taxol could be used.

The molecular component of microtubules (tubulin) is in a state of dynamic equilibrium between the polymerised and non-polymerised states. Microtubule inhibitors such as nocodazole prevent the addition of tubulin molecules to microtubules, thereby disturbing the equilibrium and leading to microtubule depolymerisation and destruction of the spindle. It is preferred to add the microtubule inhibitor a sufficient time before activation to ensure complete, or almost complete, depolymerisation of the microtubules. Twenty to thirty minutes is likely to be sufficient in most cases. A microtubule stabiliser such as taxol prevents the breakdown of the spindle and may also therefore prevent the production of multiple pronuclei. Use of a microtubule stabiliser is preferably under similar conditions to those used for microtubule inhibitors.

The microtubule inhibitor or stabiliser should remain present after activation until pronuclei formation. It should be removed thereafter, and in any event before the first division takes place.

5

In a preferred embodiment of the invention at 30-42 hours post onset of maturation (bovine and ovine, i.e. 6-18 hours post nuclear transfer) the reconstructed oocytes are placed into medium containing nocodazole (5 $\mu$ g/ml) and activated using conventional protocols. Incubation in nocodazole may be continued for 4-6 hours following the activation stimulus (dependent upon species and oocyte age).

10 According to a second aspect of the invention, there is provided a viable reconstituted animal embryo prepared by a method as described previously.

15 According to a third aspect of the invention, there is provided a method of preparing an animal, the method comprising:

- 20
- (a) reconstituting an animal embryo as described above; and
  - 25 (b) causing an animal to develop to term from the embryo; and
  - (c) optionally, breeding from the animal so formed.

Step (a) has been described in depth above.

30

The second step, step (b) in the method of this aspect of the invention is to cause an animal to develop to term from the embryo. This may be done directly or indirectly. In direct development, the reconstituted embryo from step

(a) is simply allowed to develop without further intervention beyond any that may be necessary to allow the development to take place. In indirect development, however, the embryo may be further manipulated before  
5 full development takes place. For example, the embryo may be split and the cells clonally expanded, for the purpose of improving yield.

Alternatively or additionally, it may be possible for  
10 increased yields of viable embryos to be achieved by means of the present invention by clonal expansion of donors and/or if use is made of the process of serial (nuclear) transfer. A limitation in the presently achieved rate of blastocyst formation may be due to the  
15 fact that a majority of the embryos do not "reprogram" (although an acceptable number do). If this is the case, then the rate may be enhanced as follows. Each embryo that does develop itself can be used as a nuclear donor at the 32-64 cell stage; alternatively, inner cell mass  
20 cells can be used at the blastocyst stage. If these embryos do indeed reflect those which have reprogrammed gene expression and those nuclei are in fact reprogrammed (as seems likely), then each developing embryo may be multiplied in this way by the efficiency of the nuclear  
25 transfer process. The degree of enhancement likely to be achieved depends upon the cell type. In sheep, it is readily possible to obtain 55% blastocyst stage embryos by transfer of a single blastomere from a 16 cell embryo to a preactivated "Universal Recipient" oocyte. So it is  
30 reasonable to hypothesise that each embryo developed from a single cell could give rise to eight at the 16 cell stage. Although these figures are just a rough guide, it is clear that at later developmental stages the extent of benefit would depend on the efficiency of the process at  
35 that stage.

Aside from the issue of yield-improving expediciencies, the reconstituted embryo may be cultured, *in vivo* or *in vitro* to blastocyst.

5 Experience suggests that embryos derived by nuclear transfer are different from normal embryos and sometimes benefit from or even require culture conditions *in vivo* other than those in which embryos are usually cultured (at least *in vivo*). The reason for this is not known.  
10 In routine multiplication of bovine embryos, reconstituted embryos (many of them at once) have been cultured in sheep oviducts for 5 to 6 days (as described by Willadsen, In Mammalian Egg Transfer (Adams, E.E., ed.) 185 CRC Press, Boca Raton, Florida (1982)). In the  
15 practice of the present invention, though, in order to protect the embryo it should preferably be embedded in a protective medium such as agar before transfer and then dissected from the agar after recovery from the temporary recipient. The function of the protective agar or other  
20 medium is twofold: first, it acts as a structural aid for the embryo by holding the zona pellucida together; and secondly it acts as barrier to cells of the recipient animal's immune system. Although this approach increases the proportion of embryos that form blastocysts, there is  
25 the disadvantage that a number of embryos may be lost.

If *in vitro* conditions are used, those routinely employed in the art are quite acceptable.

30 At the blastocyst stage, the embryo may be screened for suitability for development to term. Typically, this will be done where the embryo is transgenic and screening and selection for stable integrants has been carried out. Screening for non-transgenic genetic markers may also be



carried out at this stage. However, because the method of the invention allows for screening of donors at an earlier stage, that will generally be preferred.

5 After screening, if screening has taken place, the blastocyst embryo is allowed to develop to term. This will generally be *in vivo*. If development up to blastocyst has taken place *in vitro*, then transfer into the final recipient animal takes place at this stage. If  
10 blastocyst development has taken place *in vivo*, although in principle the blastocyst can be allowed to develop to term in the pre-blastocyst host, in practice the blastocyst will usually be removed from the (temporary) pre-blastocyst recipient and, after dissection from the  
15 protective medium, will be transferred to the (permanent) post-blastocyst recipient.

In optional step (c) of this aspect of the invention, animals may be bred from the animal prepared by the  
20 preceding steps. In this way, an animal may be used to establish a herd or flock of animals having the desired genetic characteristic(s).

Animals produced by transfer of nuclei from a source of  
25 genetically identical cells share the same nucleus, but are not strictly identical as they are derived from different oocytes. The significance of this different origin is not clear, but may affect commercial traits. Recent analyses of the mitochondrial DNA of dairy cattle  
30 in the Iowa State University Breeding Herd revealed associated with milk and reproductive performance (Freeman & Beitz, In Symposium on Cloning Mammals by Nuclear Transplantation (Seidel, G. E. Jr., ed.) 17-20, Colorado State University, Colorado (1992)). It remains

to be confirmed that similar effects are present throughout the cattle population and to consider whether it is possible or necessary in specific situations to consider the selection of oocytes. In the area of cattle breeding the ability to produce large numbers of embryos from donors of high genetic merit may have considerable potential value in disseminating genetic improvement through the national herd. The scale of application will depend upon the cost of each embryo and the proportion of transferred embryos able to develop to term.

By way of illustration and summary, the following scheme sets out a typical process by which transgenic and non-transgenic animals may be prepared. The process can be regarded as involving five steps:

- (1) isolation of diploid donor cells;
- (2) optionally, transgenesis, for example by transfection with suitable constructs, with or without selectable markers;
  - (2a) optionally screen and select for stable integrants - skip for micro-injection;
- (3) embryo reconstitution by nuclear transfer;
- (4) culture, in vivo or in vitro, to blastocyst;
  - (4a) optionally screen and select for stable integrants - omit if done at 2a - or other desired characteristics;
- (5) transfer if necessary to final recipient.

This protocol has a number of advantages over previously published methods of nuclear transfer:

- 1) The chromatin of the donor nucleus can be exposed to the meiotic cytoplasm of the recipient oocyte in the

absence of activation for appropriate periods of time. This may increase the "reprogramming" of the donor nucleus by altering the chromatin structure.

5        2)    Correct ploidy of the reconstructed embryo is maintained when G0/G1 nuclei are transferred.

10       3)    Previous studies have shown that activation responsiveness of bovine/ovine oocytes increases with age. One problem which has previously been observed is that in unenucleated aged oocytes duplication of the meiotic spindle pole bodies occurs and multipolar spindles are observed. However, we report that in embryos reconstructed and maintained with high MPF levels  
15       although nuclear envelope breakdown and chromatin condensation occur no organised spindle is observed. The prematurely condensed chromosomes remain in a tight bunch, therefore we can take advantage of the ageing process and increase the activation response of the  
20       reconstructed oocyte without adversely affecting the ploidy of the reconstructed embryo.

According to a fourth aspect of the invention, there is provided an animal prepared as described above.

25       Preferred features of each aspect of the invention are as for each other aspect, *mutatis mutandis*.

30       The invention will now be described by reference to the accompanying Examples which are provided for the purposes of illustration and are not to be construed as being limiting on the present invention. In the following description, reference is made to the accompanying drawing, in which:

FIGURE 1 shows the rate of maturation of bovine oocytes in vitro.

Example 1: "MAGIC" Procedure using Bovine Oocytes

5 Recipient oocytes the subject of this experimental procedure are designated MAGIC (Metaphase Arrested G1/G0 Acc<sup>pt</sup>Ing Cyt<sup>pl</sup>ast) Recipients.

10 The nuclear and cytoplasmic events during in vitro oocyte maturation were studied. In addition the roles of fusion and activation in embryos reconstructed at different ages were also investigated. The studies have shown that oocyte maturation is asynchronous; however, a population of matured oocytes can be morphologically selected at 18  
15 hours (Figure 1).

Morphological selection of oocytes

In Figure 1 ovaries were obtained from a local abattoir and maintained at 28-32°C during transport to the  
20 laboratory. Cumulus oocyte complexes (COC's) were aspirated from follicles 3-10mm in diameter using a hypodermic needle (1.2mm internal diameter) and placed into sterile plastic universal containers. The universal containers were placed into a warmed chamber (35°C) and  
25 the follicular material allowed to settle for 10-15 minutes before pouring off three quarters of the supernatant. The remaining follicular material was diluted with an equal volume of dissection medium (TCM 199 with Earles salts (Gibco), 75.0 mg/l kanamycin,  
30 30.0mM Hepes, pH 7.4, osmolarity 280 mOsmols/Kg H<sub>2</sub>O) supplemented with 10% bovine serum, transferred into an 85mm petri dish and searched for COC's under a dissecting microscope.

Complexes with at least 2-3 compact layers of cumulus cells were selected washed three times in dissection medium and transferred into maturation medium (TC medium 199 with Earles salts (Gibco), 75mg/l kanamycin, 30.0mM Hepes, 7.69mM NaHCO<sub>3</sub>, pH 7.8, osmolarity 280 mOsmols/Kg H<sub>2</sub>O) supplemented with 10% bovine serum and 1x10<sup>6</sup> granulosa cells/ml and cultured on a rocking table at 39°C in an atmosphere of 5% CO<sub>2</sub> in air. Oocytes were removed from the maturation dish and wet mounted on ethanol cleaned glass slides under coverslips which were attached using a mixture of 5% petroleum jelly 95% wax. Mounted embryos were then fixed for 24 hours in freshly prepared methanol: glacial acetic acid (3:1), stained with 45% aceto-orcein (Sigma) and examined by phase contrast and DIC microscopy using a Nikon Microphot-SA, the graph in Figure 1 shows the percentage of oocytes at MII and those with a visible polar body.

#### Activation of bovine follicular oocytes

If maturation is then continued until 24 hours these oocytes activate at a very low rate (24%) in mannitol containing calcium (Table 1a). However, removal of calcium and magnesium from the electropulsing medium prevents any activation.

Table 1a shows activation of bovine follicular oocytes matured in vitro for different periods. Oocytes were removed from the maturation medium, washed once in activation medium, placed into the activation chamber and given a single electrical pulse of 1.25kV/cm for 80µs.

Table 1a

No. of oocytes (N)	Hours post onset of maturation (hpm) [age (hrs)]	Pronuclear formation (% activation)
73	24	24.6
99	30	84.8
55	45	92.7*

\*many 2 or more pronuclei

10 Activation response of sham enucleated bovine oocytes  
 Table 1b shows activation response of *in vitro* matured bovine oocytes sham enucleated at approximately 22 hours post onset of maturation (hpm). Oocytes were treated exactly as for enucleation, a small volume of cytoplasm was aspirated not containing the metaphase plate. After manipulation the oocytes were given a single DC pulse of 1.25 KV/cm and returned to the maturation medium, at 30 hpm and 42 hpm groups of oocytes were mounted, fixed and stained with aceto-orcein. The results show the number of oocytes at each time point from five individual experiments as the number of cells having pronuclei with respect to the total number of cells.

Table 1b

EXPERIMENT	No. cells having pronuclei/ Total no. of cells	No. cells having pronuclei/ Total no. of cells
	30 hpm	42 hpm
1	1/8	-
2	0/24	0/30
3	0/21	0/22
4	0/27	0/25
5	0/19	0/1

hpm = hours post onset of maturation

Pronuclear formation in enucleated oocytes

Table 2 shows pronuclear formation in enucleated oocytes fused to primary bovine fibroblasts (24 hpm) and subsequently activated (42hpm). The results represent five separate experiments. Oocytes were divided into two groups, group A were incubated in nocodazole for 1 hour prior to activation and for 6 hours following activation. Group B were not treated with nocodazole. Activated oocytes were fixed and stained with aceto-orcein 12 hours post activation. The number of pronuclei (PN) in each parthenote was then scored under phase contrast. The results are expressed as the percentage of activated oocytes containing 1 or more pronuclei.

Table 2

	TOTAL	_1 PN	2 PN	3 PN	4 PN	>4 PN
GROUP A	52	100	0	0	0	0
GROUP B	33	45.2	25.8	16.1	3.2	9.7

The absence of an organised spindle and the absence of a polar body suggests that in order to maintain ploidy in the reconstructed embryo then only a diploid i.e. G0/G1 nucleus should be transferred into this cytoplasmic situation. Incubation of activated oocytes in the presence of the microtubule inhibitor nocodazole for 5 hours, 1 hour prior to and following the activation stimulus prevents the formation of micronuclei (Table 2) and thus when the donor nucleus is in the G0/G1 phase of the cell cycle the correct ploidy of the reconstructed embryo is maintained.

Results

These results show that:

i) these oocytes can be enucleated at 18 hours post onset of maturation (Figure 1);

5 ii) enucleated oocytes can be fused to donor blastomeres/cells in either 0.3M mannitol or 0.27M sucrose alternatively the donor the cells or nuclei can be injected in calcium free medium in the absence of any activation response;

10 iii) reconstructed embryos or enucleated pulsed oocytes can be cultured in maturation medium and do not undergo spontaneous activation;

15 iv) the transferred nucleus is seen to undergo nuclear envelope breakdown (NEBD) and chromosome condensation. No organised meiotic/mitotic spindle is observed regardless of the cell cycle stage of the transferred nucleus;

20 v) such manipulated couplets will activate at 30 hours and 42 hours with a frequency equal to unmanipulated control oocytes;

25 vi) no polar body is observed following subsequent activation, regardless of the cell cycle stage of the transferred nucleus;

viii) upon subsequent activation 1-5 micronuclei are formed per reconstructed zygote (Table 2).

30

Reconstruction of bovine embryos using "MAGIC" procedure  
In preliminary experiments this technique has been applied to the reconstruction of bovine embryos using primary fibroblasts synchronised in the G0 phase of the



cell cycle by serum starvation for five days. The results are summarised in Table 3.

Table 3 shows development of bovine embryos reconstructed by nuclear transfer of serum starved (G0) bovine primary fibroblasts into enucleated unactivated MII oocytes. Embryos were reconstructed at 24 hpm and the fused couplets activated at 42 hpm. Fused couplets were incubated in nocodazole (5 $\mu$ g/ml) in M2 medium for 1 hour prior to activation and 5 hours post activation. Couplets were activated with a single DC pulse of 1.25 KV/cm for 80 $\mu$ sec.

Table 3

EXPERIMENT NUMBER	NUMBER OF BLASTOCYSTS/ TOTAL NUMBER OF FUSED COUPLETS	% BLASTOCYSTS
1	1/30	3.3
2	4/31	12.9

Example 2: "MAGIC" Procedure using Ovine Oocytes

Similar observations to those in Example 1 have also been made in ovine oocytes which have been matured in vivo. Freshly ovulated oocytes can be retrieved by flushing from the oviducts of superstimulated ewes 24 hours after prostaglandin treatment. The use of calcium magnesium free PBS/1.0% FCS as a flushing medium prevents oocyte activation. Oocytes can be enucleated in calcium free medium and donor cells introduced as above in the absence of activation. No organised spindle is observed, multiple nuclei are formed upon subsequent activation and this may be suppressed by nocodazole treatment.

### Results

In preliminary experiments in sheep, a single pregnancy has resulted in the birth of a single live lamb. The results are summarised in Tables 4 and 5.

5

Table 4 shows development of ovine embryos reconstructed by transfer of an embryo derived established cell line to unactivated enucleated *in vivo* matured ovine oocytes. Oocytes were obtained from superstimulated Scottish blackface ewes, the cell line was established from the embryonic disc of a day 9 embryo obtained from a Welsh mountain ewe. Reconstructed embryos were cultured in the ligated oviduct of a temporary recipient ewe for 6 days, recovered and assessed for development.

15

Table 4

20	DATE OF NUCLEAR TRANSFER	PASSAGE NUMBER	NUMBER OF MORULA, BLA STOCYSTS / TOTAL NUMBER
	17.1.95	6	4/28
	19.1.95	7	1/10
	31.1.95	13	0/2
25	2.2.95	13	0/14
	7.2.95	11	1/9
	9.2.95	11	1/2
	14.2.95	12	
	16.2.95	13	3/13
30			
	TOTAL		10/78 (12.8%)

Table 5 shows induction of pregnancy following transfer of all morula/blastocyst stage reconstructed embryos to the uterine horn of synchronised final recipient blackface ewes. The table shows the total number of embryos from each group transferred the frequency of pregnancy in terms of ewes and embryos, in the majority of cases 2 embryos were transferred to each ewe. A single twin pregnancy was established which resulted in the birth of a single live lamb.

Table 5

PASSAGE NUMBER	"MAGIC"
P6	4
P7	1
P11	2
P12	0
P13	3
TOTAL MOR/BL	10
TOTAL NUMBER EWES	6
PREGNANT EWES %	1 (16.7)
FOETUSES/ TOTAL TRANSFERRED (%)	2/10 (20.0)

CLAIMS

1. A method of reconstituting an animal embryo, the process comprising transferring a diploid nucleus into an oocyte which is arrested in the metaphase of the second meiotic division without concomitantly activating the oocyte, keeping the nucleus exposed to the cytoplasm of the recipient for a period of time sufficient for the embryo to become capable of giving rise to a live birth and subsequently activating the reconstituted embryo while maintaining correct ploidy.
2. A method as claimed in claim 1, in which the animal is an ungulate species.
3. A method as claimed in claim 2, in which the animal is a cow or bull, pig, goat, sheep, camel or water buffalo.
4. A method as claimed in any one of claims 1 to 3, in which the donor nucleus is genetically modified.
5. A method as claimed in any one of claims 1 to 4, wherein the diploid nucleus is donated by a quiescent cell.
6. A method as claimed in any one of claims 1 to 5, wherein the recipient oocyte is enucleate.
7. A method as claimed in any one of claims 1 to 6, wherein nuclear transfer is achieved by cell fusion.
8. A method as claimed in any one of claims 1 to 7, wherein the animal is a cow or bull and wherein the donor

nucleus is kept exposed to the recipient cytoplasm for a period of from 6 to 20 hours prior to activation.

5 9. A method as claimed in any one of claims 1 to 8, wherein correct ploidy is maintained during activation by microtubule inhibition.

10 10. A method as claimed in claim 9, wherein microtubule inhibition is achieved by the application of nocodazole.

11. A method as claimed in any one of claims 1 to 8, wherein correct ploidy is maintained during activation by microtubule stabilisation.

15 12. A method as claimed in claim 11, wherein microtubule stabilisation is achieved by the application of taxol.

13. A method of preparing an animal, the method comprising:

- 20
- (a) reconstituting an animal embryo as claimed in any preceding claim;
  - (b) causing an animal to develop to term from the embryo; and
  - 25 (c) optionally, breeding from the animal so formed.

14. A method as claimed in claim 13, wherein the animal embryo is further manipulated prior to full development of the embryo.

30 15. A method as claimed in claim 14, wherein more than one animal is derived from the embryo.

16. A reconstituted animal embryo which is capable of giving rise to a live birth and is prepared by a method as claimed in any one of claims 1 to 12.

5 17. An animal prepared by a method as claimed in any one of claims 13 to 15.

18. An animal developed from an embryo as claimed in claim 16.

1/1

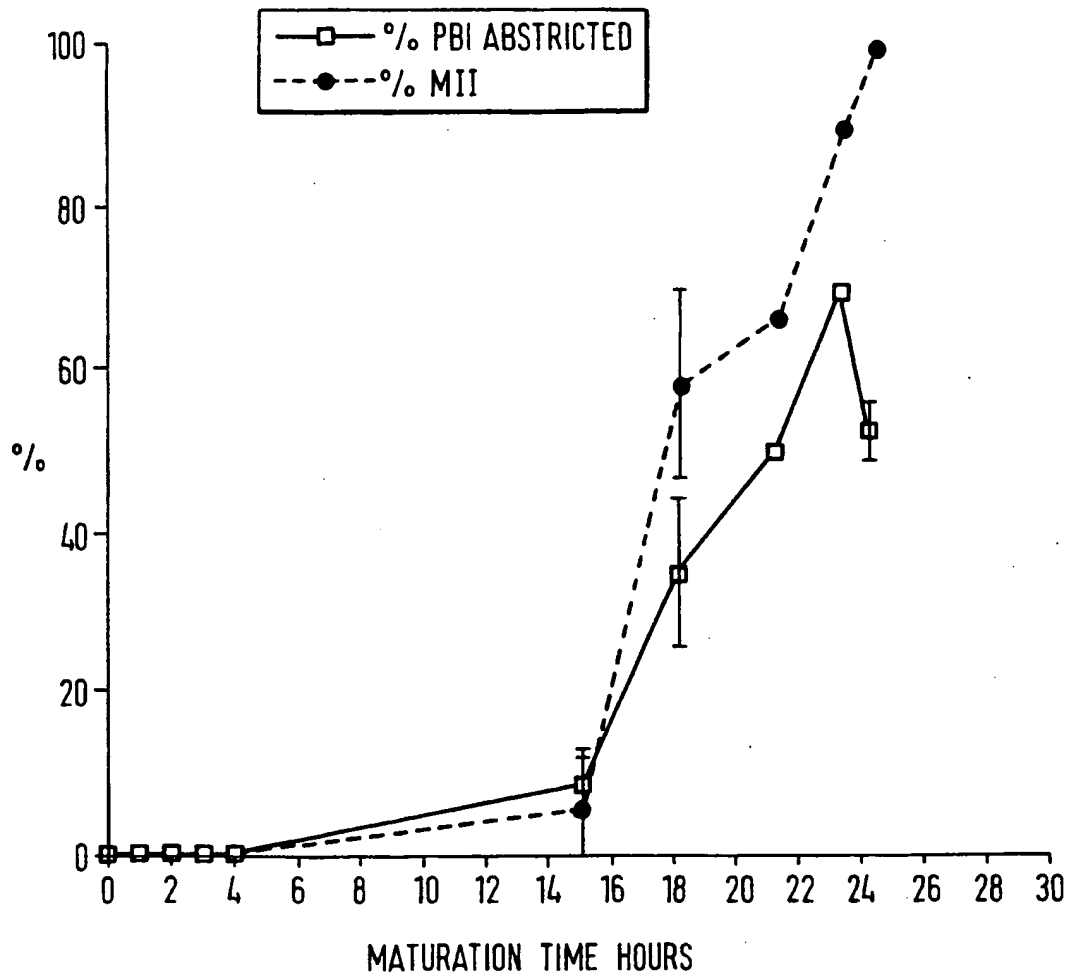


FIG. 1

## INTERNATIONAL SEARCH REPORT

International Application No

PC/GB 96/02098

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A01K67/027 A61K35/54

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A01K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF CELL SCIENCE, vol. 69, 1984, pages 19-34, XP002016866 CZOLOWSKA, R. ET AL.: "Behaviour of thymocyte nuclei in non-activated and activated mouse oocytes"	1
Y	see page 27, line 1 - page 33, line 3; table 1	1-3
X	BIOLOGY OF REPRODUCTION, vol. 43, no. 5, November 1990, pages 877-884, XP000607321 COLLAS, P. & ROBL, J.M.: "Factors affecting the efficiency of nuclear transplantation in the rabbit embryo" cited in the application see page 879, paragraph RESULTS - page 882, column 2; tables 1-3,5	1

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

25 October 1996

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## INTERNATIONAL SEARCH REPORT

International Application No

PC1/GB 96/02098

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>BIOLOGY OF REPRODUCTION, vol. 49, no. 5, 1 November 1993, pages 933-942, XP000604579 CAMPBELL, K.H.S. ET AL.: "Nuclear-cytoplasmic interactions during the first cycle of nuclear transfer reconstructed bovine embryos: implications fro desoxyribonucleic acid replication and development" cited in the application see page 941, column 2</p>	1-3
A	<p>MOLECULAR REPRODUCTION AND DEVELOPMENT, vol. 39, no. 2, 1 October 1994, pages 147-152, XP000604559 OTAEGUI, P.J. ET AL.: "Transfer of nuclei from 8-cell stage mouse embryos following use of nocodazole to control the cell cycle" see the whole document</p>	9,10
A	<p>WO,A,95 03795 (US HEALTH ;KINSELLA JAMES L (US); SOLLOTT STEVEN J (US)) 9 February 1995 see claims</p>	11,12
A	<p>WO,A,94 06422 (US HEALTH) 31 March 1994 see claims</p>	11,12
A	<p>NATURE, vol. 320, no. 6, March 1986, LONDON GB, pages 63-65, XP000569670 WILLADSEN, S.M.: "Nuclear transplantation in sheep embryos" cited in the application see the whole document</p>	1-3
A	<p>THE WISTAR INSTITUTE SYMPOSIUM MONOGRAPH, vol. 9, 1 January 1969, pages 19-33, XP000607322 GRAHAM, C.F.: "The fusion of cells with one- and two-cell mouse embryo" cited in the application see the whole document</p>	1
P,X	<p>THERIOGENOLOGY, vol. 45, no. 1, 7 January 1996, page 287 XP000605648 CAMPBELL, K.H.S. ET AL.: "Live lambs by nuclear transfer from an established cell line" see abstract</p>	1-3,5-7, 13,16-18

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PL 1/GB 96/02098

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO-A-9406422	31-03-94	AU-A- 5135793 CA-A- 2145190 EP-A- 0661969 JP-T- 8501560 US-A- 5496846	12-04-94 31-03-94 12-07-95 20-02-96 05-03-96